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DEPARTAMENTO DE CIÊNCIA DE ALIMENTOS

ESTUDOS DE OBTENÇÃO DE BIOAROMAS PELA BIOTRANSFORMAÇÃO DE COMPOSTOS TERPÊNICOS

TESE DE DOUTORADO EM CIÊNCIA DE ALIMENTOS

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APRESENTAÇÃO

Este manuscrito faz parte da tese do candidato Juliano Lemos Bicas para obtenção de título de Doutor em Ciência de Alimentos. Os experimentos aqui apresentados foram efetuados no Laboratório de Bioaromas do Departamento de Ciência de Alimentos da Faculdade de Engenharia de Alimentos de Universidade Estadual de Campinas (Unicamp) entre os meses de agosto de 2005 e dezembro de 2008, sob orientação da Profa. Dra. Gláucia Maria Pastore e com financiamento da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (bolsa de estudo) e da Fundação de Amparo a Pesquisa do Estado de São Paulo (projeto de pesquisa n° 06/56824-8). Parte do trabalho foi desenvolvido no Laboratoire de Génie Chimie et Biochimie da Polytech'Clermont-Ferrand, Université Blaise Pascal, na cidade de Clermont-Ferrand (França), entre os meses de agosto de 2007 e fevereiro de 2008, sob supervisão do Prof. Dr. Christian Larroche e com apoio financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (bolsa Doutorado Sanduíche, processo n° 0725/07-2).

O texto foi estruturado em sete capítulos, na forma de artigos, nos quais serão abordados (i) um estudo bibliográfico sobre o principal sujeito da tese (bio-oxidação de terpenos); (ii) o isolamento e a seleção de microrganismos com potencial interesse para a biotransformação de terpenos; (iii) a produção de α-terpineol e (iv) sua caracterização e coprodução de lipase alcalina/α-terpineol pelo fungo filamentoso *Fusarium oxysporum*; (v) a descrição de vias metabólicas para determinados monoterpenos em linhagens bacterianas de *Pseudomonas*; (vi) um processo com elevado rendimento e produtividade de α-terpineol, empregando uma linhagem bacteriana de *Sphingobium* sp.; (vii) e a avaliação do potencial bioativo de alguns dos principais bioaromas obtidos por biotransformação do limoneno, especialmente o α-terpineol. Os capítulos

I, II, V e VI foram mantidos conforme o original, em inglês, o que garante maior acessibilidade da tese para a comunidade científica internacional. Os demais foram escritos em portugês para garantir o mínimo exigido pela Comissão de Pós-graduação da Faculdade de Engenharia de Alimentos.

RESUMO GERAL

O objetivo do presente trabalho foi efetuar estudos de biotransformação de substratos terpênicos para a obtenção de compostos de aromas naturais, ou bioaromas, enfatizando os processos bioquímicos envolvidos nos procedimentos empregados e a otimização da produção para possíveis aplicações industriais. Assim, o estudo se iniciou com o isolamento de quase 300 linhagens, das quais 121 mostraram-se resistentes a concentrações de 2% de *R*-(+)-limoneno e 70 foram capazes de utilizar este substrato como única fonte de carbono. Dentre todas as linhagens potencialmente degradantes do *R*-(+)-limoneno, nenhuma mostrou acúmulo significativo de metabólito de interesse em concentrações que justificassem estudos de otimização.

A seguir, o método de Superfície de Resposta foi empregado para otimizar os principais parâmetros do processo de produção de *R*-(+)-α-terpineol a partir do *R*-(+)-limoneno pelo fungo *Fusarium oxysporum* 152b. Dentre os 10 parâmetros analisados (concentração de glicose, peptona, extrato de malte e de levedura no meio de biotransformação; concentração de *R*-(+)-limoneno; concentração de biosurfactantes; temperatura; agitação; pH; tamanho do inóculo), três (concentração de substrato, temperatura e agitação) influenciaram significativamente ($p < 0,10$) a produção de *R*-(+)-α-terpineol, dentro das faixas estudadas. A otimização dessas variáveis por um Delineamento Composto Central Rotacional revelou que as condições ótimas para a biotransformação foram de 0,5% de *R*-(+)-limoneno, 26 °C e 240 rpm, resultando em uma concentração de cerca de 2,4 $g.L^{-1}$ de *R*-(+)-α-terpineol ao final de 72 h de processo. Aproveitando-se do fato de essa linhagem fúngica ser reconhecida pela produção de lipase

alcalina, um sistema integrado de produção foi posteriormente proposto a fim de explorar todo potencial biotecnológico do microrganismo. Assim, a biomassa resultante da produção de lipase, antes descartada, foi avaliada quanto à preservação da atividade de biotransformar o *R*-(+)-limoneno. Os resultados demonstraram ser possível a coprodução de lipase/*R*-(+)-α-terpineol, apesar de que o rendimento máximo do bioaroma foi cerca de 50% inferior quando comparado ao do procedimento convencional.

Os estudos com duas linhagens bacterianas (*Pseudomonas rhodesiae* CIP 107491 e *P. fluorescens* NCIMB 11671) para a bioconversão de alguns monoterpenos indicaram a presença de uma via metabólica envolvendo β-pineno, α-pineno, α-pineno oxido, isonovalal e ácido dimetil pentanóico para ambas espécies, além de outras duas vias de degradação do limoneno para *P. fluorescens*. Nesse caso, a bactéria usava o limoneno como única fonte de carbono e energia, passando por limoneno-1,2-diol, e também hidroxilava este substrato na posição 8 formando *R*-(+)-α-terpineol como forma de diminuir a toxicidade do substrato (metabolismo de xenobióticos). Essa última via ocorria em ausência de cofatores graças à ação de uma hidratase enantioespecífica capaz de converter anaerobicamente *R*-(+)-limoneno a *R*-(+)-α-terpineol e *S*-(–)-limoneno a *S*-(–)-α-terpineol em meios bifásicos, empregando *n*-hexadecano como fase orgânica. Foi posteriormente demonstrado que os rendimentos e produtividade poderiam ser significativamente elevados e que a produção poderia ser mais que duplicada (de ~10 para ~25 $g.L^{-1}$) com o uso de fases orgânicas não convencionais, como óleos vegetais.

Finalmente, estudos preliminares de avaliação do potencial bioativo do principal produto relatado nessa tese demonstraram que o α-terpineol revelou uma elevada capacidade de absorção de radical de oxigênio (ORAC) e atividade antiploriferativa contra cinco linhagens de células

cancerosas, apesar da baixa atividade de captura de radical DPPH. Esses resultados abrem precedentes para que pesquisas *in vivo* sejam consideradas a fim de determinar o potencial funcional desse bioaroma, algo ainda praticamente inexplorado.

ABSTRACT

The objective of the present work was to study the biotransformation of terpene substrates to obtain natural flavor compounds (bioflavors), focusing the biochemical processes involved in the procedures investigated and optimization of production for possible industrial applications. Therefore, the study started with the isolation of more than 300 wild strains followed by the selection of 121 capable of resisting to 2% ($v.v^{-1}$) of *R*-(+)-limonene and 70 that could use this terpene as sole carbon and energy source. None of the strains tested showed accumulation of intermediate metabolites in levels that justified further optimization studies.

Subsequently, the Response Surface Methodology was employed to optimize the main parameters of the process of biotransformation of *R*-(+)-limonene to *R*-(+)-α-terpineol by the fungal strain *Fusarium oxysporum* 152b. Only three (*R*-(+)-limonene concentration, temperature and agitation) of the ten parameters tested (concentration of glucose, peptone, malt extract and yeast extract; substrate concentration; biosurfactant concentration; temperature; agitation; pH; inoculum size) influenced significantly ($p < 0.1$) the *R*-(+)-α-terpineol production. The optimization of these variables applying a Central Composite Design revealed that the optimal biotrasformation conditions were 0.5% of *R*-(+)-limonene, 26 °C and 240 rpm, resulting in a *R*-(+)-α-terpineol concentration close to 2,4 $g.L^{-1}$ after a 72 h. Since this fungus has been recognized for its high alkaline lipase production, an integrated process was proposed to explore the full biotechnological potential of this microorganism. Therefore, the biomass resulting from the lipase production, which was previously discharded, was tested to evaluate the preservation of *R*-(+)-limonene-biotransformation activity. The results have shown that the co-production of

lipase/*R*-(+)-α-terpineol was feasible, although the maximal yield of the bioflavor was approximately 50 % lower when compared to the conventional process.

The studies with two pseudomonad strains (*Pseudomonas rhodesiae* CIP 107491 e *P. fluorescens* NCIMB 11671) for the conversion of some monoterpenes indicated the presence of one metabolic route involving β-pinene α-pinene α-pinene oxide, isonovalal and dimethyl pentanoic acid for both species, besides two other pathways for the degradation of limonene by *P. fluorescens*. In this case, the bacterium used the substrate as sole carbon and energy source, with limonene-1,2-diol as intermediate, and also hydroxylated limonene in the position 8 to *R*-(+)-α-terpineol as a detoxifying strategy (xenobiotic metabolism). The last pathway occurred in the absence of cofactors due to the action of an enantiospecific hydratase capable of converting anaerobically *R*-(+)-limonene to *R*-(+)-α-terpineol and *S*-(–)-limonene to *S*-(–)-α-terpineol in biphasic mediums, employing *n*-hexadecane as organic phase. It was later demonstrated that the yields and productivities could be significantly enhanced and that the final concentration of the product could be more than duplicated (from ~10 to ~25 $g.L^{-1}$) if unconventional organic phases (vegetable oils) were used.

Finally, preliminary studies evaluating the bioactive potential of the main product reported in this thesis have revealed that the α-terpineol demonstrated an oxygen radical absorbance capacity (ORAC) and an antiproliferative activity against five cancer lines, although the DPPH radical scavenging activity was low. These results encourages *in vivo* research to determine the functional potential of this bioflavor, something practically unexplored.

INTRODUÇÃO

1. O agronegócio brasileiro: uma oportunidade para produções biotecnológicas rentáveis

O agronegócio é o principal setor da economia brasileira, responsável por 33% do Produto Interno Bruto (PIB), 42% das exportações totais e 37% da geração de empregos, segundo o Ministério da Agricultura (MAPA, 2008). O mesmo Ministério calcula que, entre os anos de 1998 a 2003, a taxa de crescimento do PIB agropecuário foi de 4,67% ao ano e, no último ano, as exportações de produtos agropecuários renderam ao Brasil US$ 36 bilhões gerando um superávit de US$ 25,8 bilhões. O fato é que, atualmente, o país é um dos líderes mundiais na produção e exportação de vários produtos agropecuários. É o primeiro produtor e exportador de café, açúcar, álcool e sucos de frutas, além de liderar as vendas externas de soja, carne bovina, carne de frango, tabaco, couro e calçados de couro.

Com uma fruticultura diversificada, o Brasil é o maior produtor mundial de frutas cítricas, destacando-se por sua expressiva produção de laranjas. O Estado de São Paulo responde por cerca de 80% desta produção que, no ano de 2007, foi de pouco mais de 18,2 milhões de toneladas (FAO, 2008). A indústria citrícola brasileira transforma em suco mais de 75% do total de frutas cítricas produzidas, sendo que de 45 a 50% do peso das laranjas processadas são "perdidos" sob forma de resíduo industrial. No Brasil, essa fração vem sendo aproveitada basicamente como polpa cítrica "peletizada" que é exportada para a Europa desde o início da década de 1970 (CONCEIÇÃO, 1998). Óleo de laranja e D-limoneno também são exportados

pelo Brasil em um volume de 30 a 40 mil toneladas anualmente (ABECITRUS, 2008). Da mesma forma, a indústria de papel e celulose gera grandes quantidades do subproduto terebentina, cuja produção mundial em 1995 foi de cerca de 330 mil toneladas (SWIFT, 2004).

Na verdade, a alta geração de resíduos é uma realidade em todos os setores agropecuários, como é o caso da fabricação de queijo, na qual cerca de 90% do leite processado é transformado em soro. Entretanto, sabe-se que os resíduos agrícolas, muitas vezes vistos como subprodutos inaproveitáveis, podem ser utilizados no desenvolvimento de produtos nobres. O óleo de laranja e a terebentina, resíduo da indústria de papel e celulose, são exemplos de subprodutos ricos em terpenos (*R*-(+)-limoneno e α-pineno, respectivamente) muitas vezes empregados na síntese de solventes em tintas, resinas, adesivos ou inseticidas. Contudo, de acordo com Swift (2004), matérias primas terpênicas devem ser vistas como fontes naturais e sustentáveis para alicerçar a indústria de química fina, sobretudo no que se refere à produção de aromas. Dessa forma, a biotecnologia surge como uma promissora alternativa para a agroindústria não só por aumentar rendimentos e melhorar a qualidade de seus processos, mas também por fornecer um arsenal tecnológico capaz de utilizar o potencial destes resíduos no desenvolvimento de produtos de alto valor agregado.

2. Terpenos

Um grande número de compostos de aroma possui uma estrutura que pode ser decomposta em unidades de isopreno (C_5H_{10}). Esta particularidade representa a característica comum a todos os terpenos, que, portanto, apresentam a formula geral $(C_5H_{10})_n$. Assim, os compostos terpênicos podem ser classificados, quanto ao número de carbonos, como

hemiterpenos (n = 1), monoterpenos (n = 2), sesquiterpenos (n = 3), diterpenos (n = 4), triterpenos (n = 6), tetraterpenos (n = 8), também conhecidos por carotenos, e politerpenos (n > 8), as chamadas borrachas. Para monoterpenos, outra classificação se baseia na ciclização de sua cadeia carbônica. Assim, eles podem ser monoterpenos acíclicos (moléculas abertas), monocíclicos, bicíclicos ou tricíclicos. Por definição, o termo "terpeno" designa os representantes da família dos hidrocarbonetos, sem grupos funcionais na molécula. Já "terpenóides" se refere aos terpenos oxidados, como álcoois, aldeídos, ácidos, cetonas ou epóxidos terpênicos (FONTANILLE, 2002).

Os terpenos são metabólitos secundários de plantas, produzidos, em parte, para defesa contra microrganismos e insetos. Até há pouco tempo, acreditava-se que esses compostos eram derivados da via do mevalonato. No entanto, algumas contradições foram observadas, levando à descoberta de novas vias biossintéticas, independentes do mevalonato (EISENREICH; ROHDICH; BACHER, 2001; ROHMER, 1999). Devido a suas propriedades organolépticas diferenciadas, certos monoterpenos são utilizados em fragrâncias e como ingredientes em alimentos. Os terpenos e terpenóides mais simples (mono e sesquiterpenos/terpenóides) são os principais constituintes dos óleos essenciais e são amplamente utilizados na indústria de aromas. Já os di e triterpenos são menos voláteis e podem ser obtidos a partir de gomas e resinas vegetais (TRUDGILL, 1986). Os carotenos, por sua vez, e apresentam comumentum sistema de duplas ligações conjugadas, que os tornam coloridos por absorverem radiações com comprimentos de onda na região do visível.

3. Aroma

Produtos alimentares contêm mais de 6200 compostos voláteis já identificados (BERGER, 1995), os quais podem ser percebidos seja por via nasal direta (odor) ou por via retronasal quando o alimento está no interior da boca (aroma) (LINARES, 2008). Compostos de aroma são moléculas de baixo peso molecular (em geral menores que 400 daltons) capazes de estimular as células receptoras do epitélio olfatório da cavidade nasal, sendo que dessas interações resultam uma quantidade quase incontável de notas aromáticas. O gosto, por outro lado, possui um número limitado de sensações (doce, salgado, ácido, amargo e o controverso umami) que decorrem de um estímulo por moléculas não voláteis aos receptores distribuídos nas papilas gustativas da língua. A união das percepções de gosto e de aroma dão origem ao sabor especifico de um determinado produto. Assim sendo, o aroma é um dos principais atributos de alimentos, bebidas e cosméticos e, portanto, estes compostos são frequentemente empregados para reforçar ou melhorar a percepção sensorial de tais produtos. Os aromas não apresentam nenhuma função nutritiva ou vitamínica a um alimento. No entanto, diversas bioatividades (antimicrobiana e anticarcinogênica, por exemplo) têm sido relatadas (BERGER, 1995).

Compostos de aroma podem ser obtidos de fontes vegetais e animais bem como sintetizados química ou biologicamente. Eles não apresentam uma função química específica: podem ser hidrocarbonetos, álcoois, cetonas, aldeídos, ácidos, ésteres ou lactonas (ésteres cíclicos), éteres *etc.* (Figura 1). No entanto, como pode ser observado, a classe química dos terpenos congrega os principais compostos de aromas naturalmente presentes nos vegetais.

Em geral, os compostos de aroma estão presentes em quantidades ínfimas na composição dos alimentos, usualmente por volta de 50 ppm, já que muitos possuem um limiar de detecção (*threshold*) na ordem de partes por bilhão (ppb) (FONTANILLE, 2002). Produtos com aromas

complexos, como é o caso do café, cacau, vinho, cerveja *etc*., apresentam mais de 500 compostos voláteis que, juntos, são responsáveis pela formação do seu aroma característico (BERGER, 1995). Em alguns casos, porém, o aroma de determinados alimentos pode resultar da ação de basicamente uma única substância, chamada "composto de impacto", que lembra o aroma característico da matriz. Um exemplo é o acetato de isopropila, composto de impacto da banana. Nesse contexto, uma técnica analítica bastante eficaz para a avaliação do perfil aromático de um alimento é a Cromatografia Gasosa-Olfatometria (CG-O), capaz de separar as frações voláteis que são avaliadas por um provador treinado e posteriormente identificadas por espectrometria de massas (CG-EM) (WAGNER, 2008).

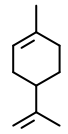
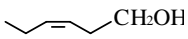
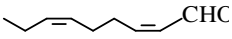
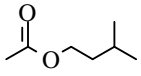
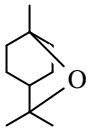
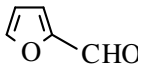
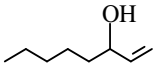
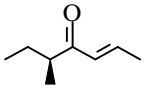
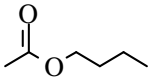
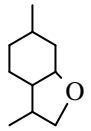
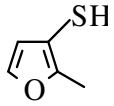
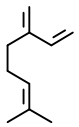
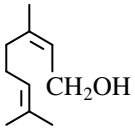
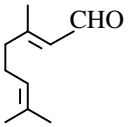
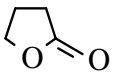
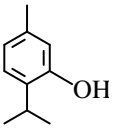
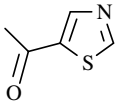
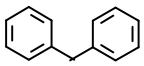
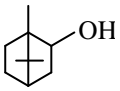
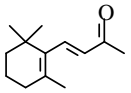
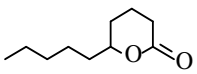
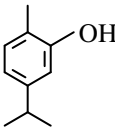
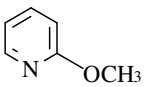
Hidrocarbonetos	Álcoois	Compostos Carbonílicos	Ésteres e Lactonas	Éteres e fenóis	Outros
					
Limoneno (cítrico)	3-hexen-1-ol (ervas)	2,6-nonadien-1-al (pepino)	Acetato de isoamila (banana)	1,8-cineole (eucalipto)	Furfural (caramelo)
					
α-pineno (coníferas)	1-octen-3-ol (cogumelo)	5-metil-(E)-2-hepten-4-ona (avelã)	Acetato de butila (maçã)	3,9-oxi-1-*p*-menteno (anis)	2-metil-3-furanotiol (rosbife)
					
Mirceno (cítrico, fresco)	Nerol (limão)	Geranial (limão)	*γ*-butirolactona (doce, amanteigado)	Timol (tomilho)	5-acetil-tiazol (carne grelhada)
					
Difenil metano (gerânio)	Borneol (canforado)	*β*-ionona (frutal)	*δ*-decalactona (pêsego)	Carvacrol (orégano)	2-acetil-piridina (crosta de pão)

Figura 1. Exemplos de compostos de aroma de diferentes funções químicas e suas respectivas notas sensoriais (BAUER; GARBE; SURBURG, 200l; FONTANILLE, 2002).

4. Obtenção de Compostos de Aroma

Há basicamente três métodos para se obter compostos de aroma: (i) extração diretamente da natureza, (ii) transformações químicas ou (iii) transformações por via biotecnológica, que incluem biotransformações microbianas e enzimáticas, síntese *de novo* e engenharia genética (FRANCO, 2004).

A obtenção de aromas naturais tem sido feita usualmente através de extração de vegetais. Entretanto, este método apresenta desvantagens como a baixa concentração dos compostos de interesse, as extrações serem extremamente caras e dependentes de fatores sazonais, climáticos e políticos além dos eventuais problemas ecológicos que podem surgir com o extrativismo.

As transformações químicas geram rendimentos aparentemente satisfatórios, entretanto muitas vezes não apresentam régio- ou enantioseletividade ao substrato, resultando em misturas de produtos. Além disso, os compostos oriundos de processos químicos só podem ser rotulados como “artificial” ou “idêntico ao natural”.

Já a produção por via biotecnológica emerge como uma alternativa atrativa para a produção de aromas, uma vez que ocorre em condições brandas, apresenta elevada régio- e enantioseletividade, não gera resíduos tóxicos e os produtos obtidos podem ser classificados como “naturais”. Ademais, existem certos compostos só possíveis de serem sintetizados por via biotecnológica.

5. Transformações de terpenos para obtenção de compostos de aroma

As transformações de terpenos para a obtenção de compostos de aroma podem ser efetuadas basicamente por métodos químicos ou biológicos. Pelos mesmos motivos já citados, a biotransformação tem se mostrado uma técnica cada vez mais interessante do ponto de vista industrial, apesar de que a instabilidade química do precursor e do produto, a baixa solubilidade do precursor, a elevada volatilidade do precursor e do produto, a alta toxicidade do precursor e do produto e as baixas taxas de biotransformação ainda representam importantes desafios a serem superados (KRINGS & BERGER, 1998).

O próximo capítulo apresenta uma revisão com os principais avanços das metodologias de oxidação de terpenos de interesse para a indústria de aromas.

CAPÍTULO 1

OXIDATION OF TERPENES: A REVIEW

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1. Introduction

The terpenes are secondary metabolites of plants that are produced, in part, as a defense against microorganisms and insects in addition to their pollinator-attractive properties (GERSHENZON; DUDAREVA, 2007). In mammals, terpenes contribute to stabilizing cell membranes, participate in metabolic pathways and act as regulators in some enzymatic reactions (DE CARVALHO; DA FONSECA, 2006a). The simpler terpenes (mono and sesquiterpenes) are the major constituents of essential oils and are widely used in the perfumery industry, while di and triterpenes are less volatile and are obtained from plant gums and resins (TRUDGILL, 1986). Carotenes are synthesized by bacteria, algae, fungi and by green plants and comprise more than 600 known structures (SANDMANN, 2001). The most important terpenes and their oxygenated derivates (terpenoids) cited in this study may be seen in Figure 2, 3 and 4.

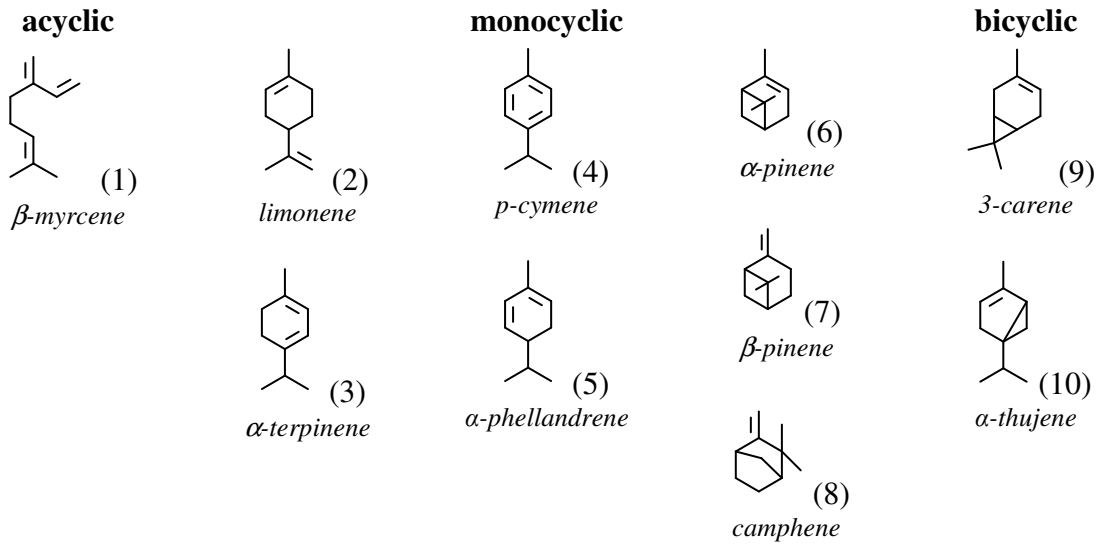


Figure 2. Main monoterpenes cited in this manuscript.

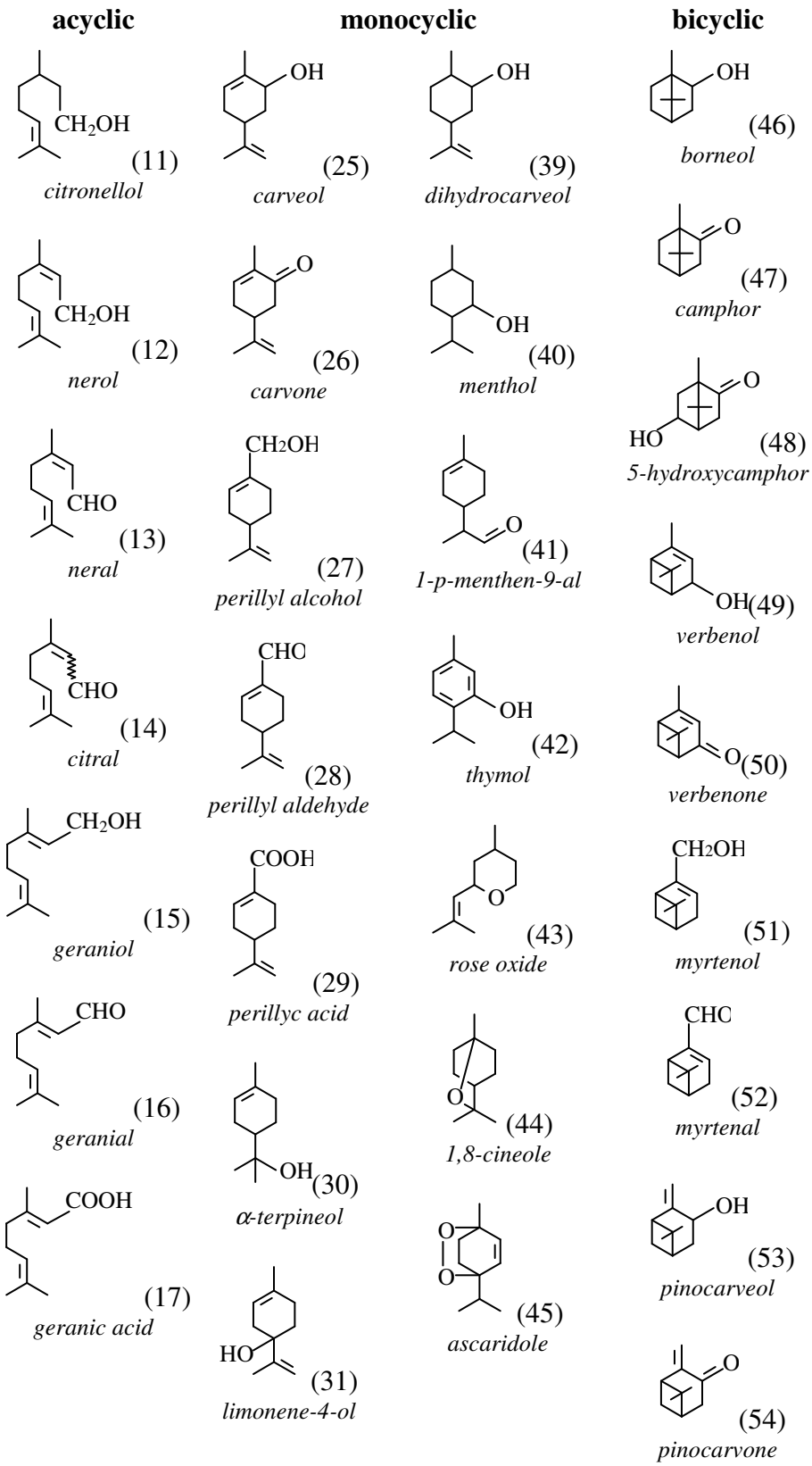


Figure 3 continue on the next page…

Figure 3. Continuation…

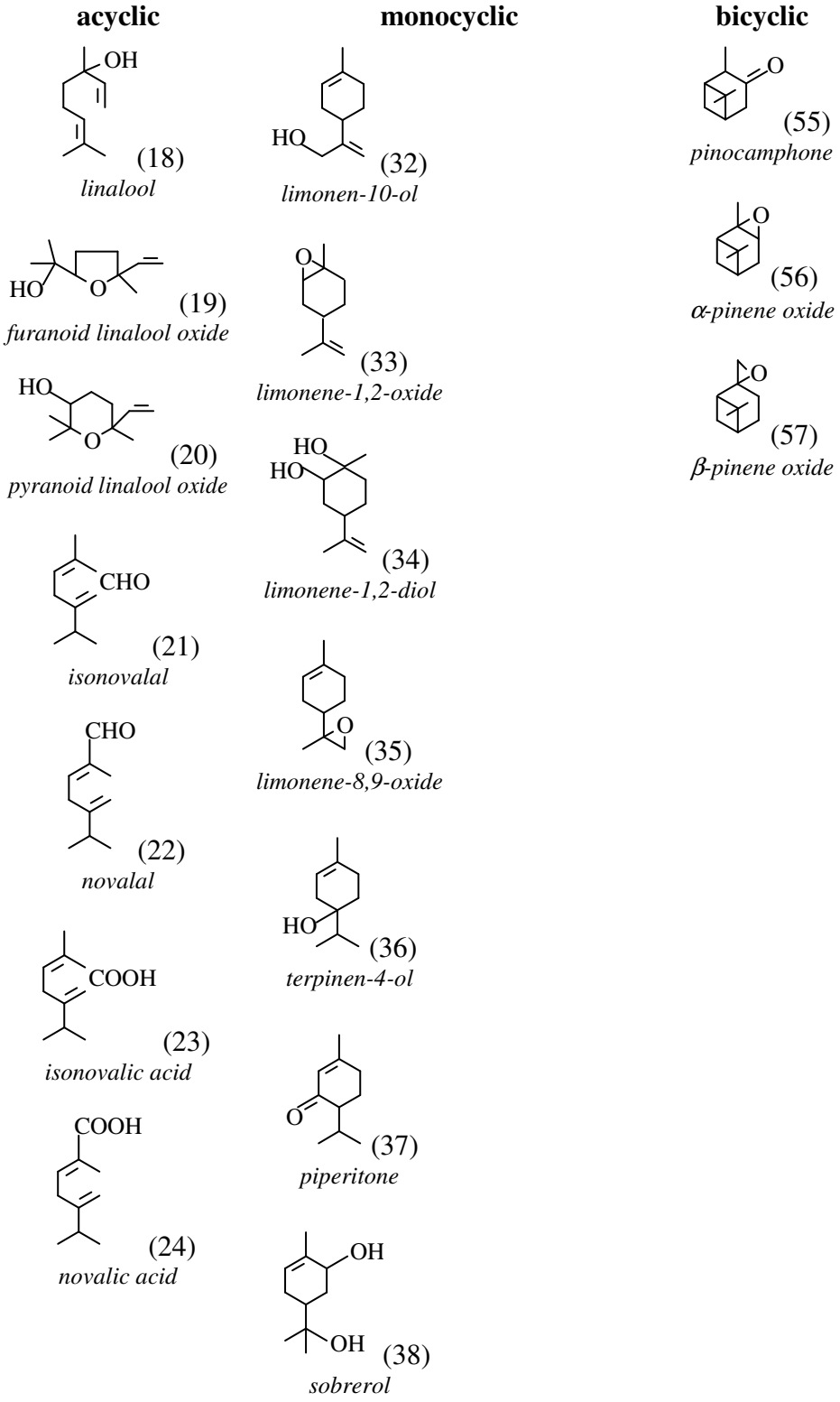


Figure 3. Main monoterpenoids cited in this manuscript.

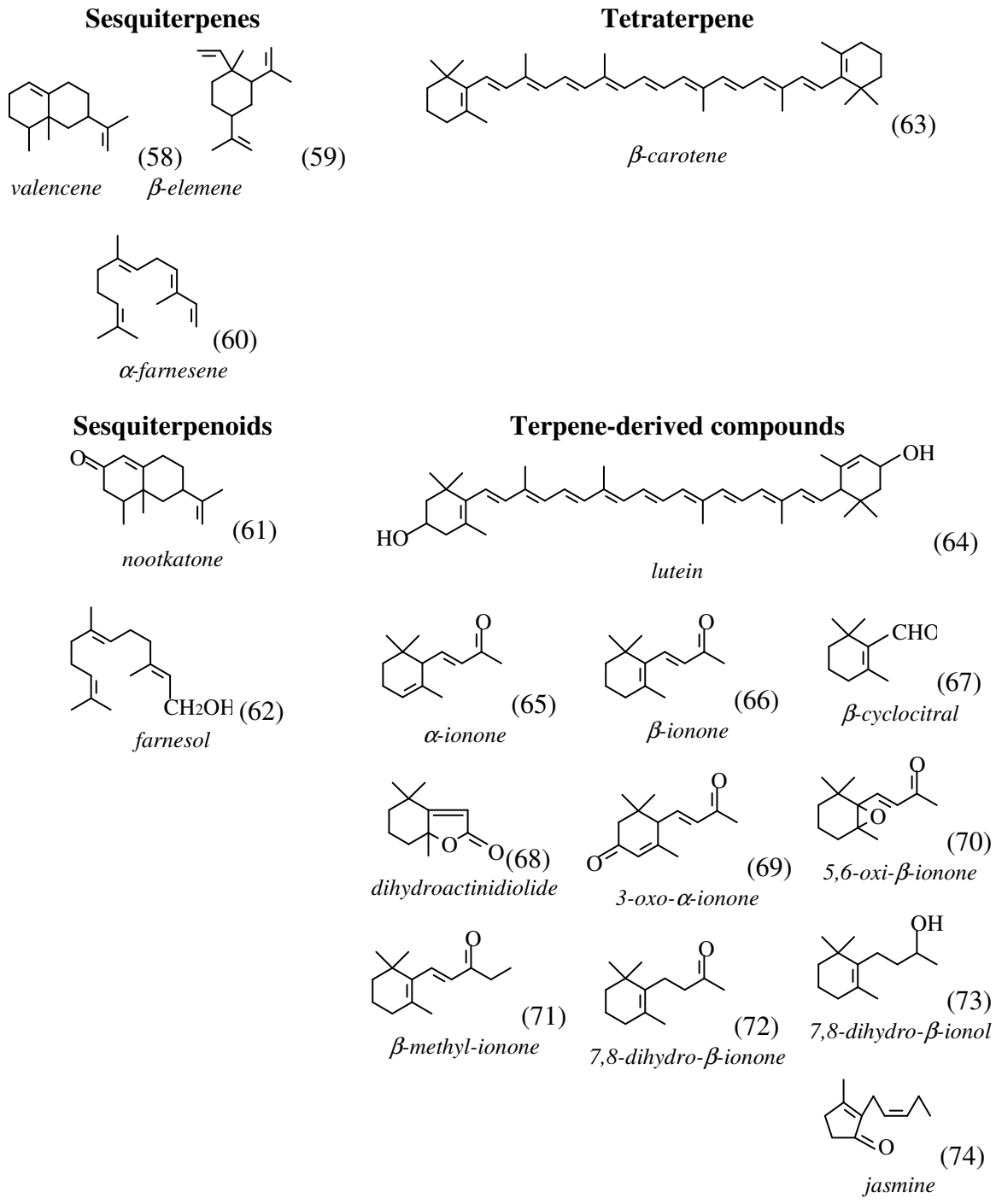


Figure 4. Other terpenes and oxygenated terpene-derived compounds cited in this study.

Terpenes are a good starting material for the synthesis of many fine chemicals due to their similar carbon skeleton. *R*-(+)-limonene **(2)**, for example, is the most abundant monocyclic

monoterpene in nature and it represents more than 90% of the orange peel oil, thus it is an inexpensive precursor (BAUER; GARBE; SURBURG, 2001; FENAROLI, 1971; MATTHEWS; BRADDOCK, 1987). The oxygenated derivatives of limonene, *eg.* carveol **(25)**, carvone **(26)**, perillyl alcohol **(27)**, menthol **(40)** and α-terpineol **(30)**, are recognized for their pleasant fragrances (RAVINDRANATH, 1983), and some of them also present bioactivity against certain types of tumor cells, not only preventing the formation or progression of cancer, but also regressing existing malignant tumors (CROWELL, 1999; JUN; JEONG; HO, 2006). α- **(6)** and β-pinene **(7)**, in turn, are found in high concentrations in turpentine, a paper and pulp industry residue, and are therefore, also available in bulk at a low price. These bicyclic monoterpenes are precursors of important flavor compounds, such as terpineols, borneol **(46)**, camphor **(47)**, citronellol **(11)**, geraniol **(15)**, menthol **(40)**, verbenol **(49)** and verbenone **(50)** (FENAROLI, 1971; BAUER; GARBE; SURBURG, 2001). The tetraterpene β-carotene **(63)**, an orange pigment found mainly in tropical vegetables, is a precursor of norisoprenoid ionones, molecules responsible for desirable fruity and floral flavors (BALDERMANN; NAIM; LEICHMANN, 2005; BAUER; GARBE; SURBURG, 2001). Volatile carotenoid breakdown products have been long known as important flavor compounds (WINTERHALTER; ROUSEFF, 2001).

Of the approximately 6,500 known flavors, only 300 are commonly used. At present, 50 to 100 are produced by microbial fermentation, while the rest are mainly obtained by chemical synthesis (SCRAGG, 2007). The scientific literature contains many examples of reviews about the chemistry of monoterpenoids (GRAYSON, 2000), the chemical reactions of terpenes to produce flavors (SWIFT, 2004) and other fine chemicals (MONTEIRO; VELOSO, 2004), the biotransformation of limonene (DUETZ *et al*., 2003; MARÓSTICA JR.; PASTORE, 2007a) and other terpenes (DE CARVALHO; DA FONSECA, 2006a; DEMYTTENAERE, 2001;

TRUDGILL, 1990; VAN DER WERF; DE BONT; LEAK, 1997) and natural flavor production via biocatalysis (GIRI *et al*., 2001; HAGEDORN; KAPHAMMER, 1994; JANSSENS *et al.*, 1992; LOMASCOLO *et al*., 1999; SCHRADER *et al.*, 2004; SERRA; FUGANTI; BRENNA, 2005; WELSH; MURRAY; WILLIAMS, 1989). However, no reference was found of a paper that congregates all these subjects. This chapter discusses the methods developed till present days for terpene oxidation in the production of molecules that attract great interest by the flavor industry, especially the monoterpenoid and norisoprenoid natural flavor compounds produced *via* microbial biotransformation (bioflavors).

2. Chemical Transformations

One of the most extensively studied reactions involving olefins is nitrosochlorination (PISANCHYN, 1976). The first description of the transformation of terpenes using gaseous nitrosyl chloride was about 130 years ago (TILDEN; LOND, 1875; TILDEN; SHENSTONE, 1877). In the early 1950s, Royals and Horne Jr. (1951) applied the nitrosyl chloride method to produce *R*-(–)-carvone **(26)** as the sole product from *R*-(+)-limonene **(2)**, with an overall yield of 56-60% (Figure 5). Years later, a similar procedure was followed for the preparation of carvone **(26)** from orange oil (ROTHENBERGER; KRASNOFF; ROLLINS, 1980). Some other terpene nitrosochlorination processes and their variants have also been studied and patented (DERFER; KANE; YOUNG, 1966; MAGALHAES; KOKETSU; WILBERG, 1983; MULDER; VAN HELDEN, 1979; NAKAI; HARADA, NISHIMURA, 1978; REITSEMA, 1957). This is an industrially important methodology for the preparation of the distinguished flavor compound carvone.

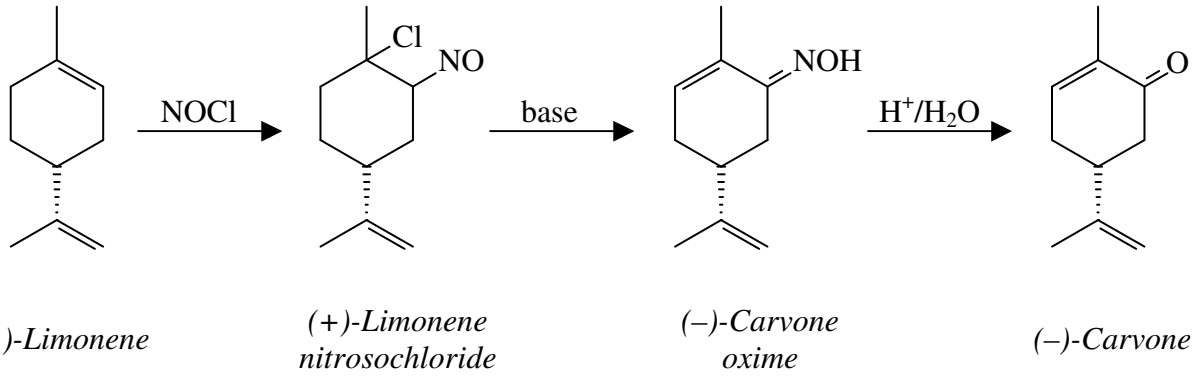


Figure 5. Oxidation of *R*-(+)-limonene to *R*-(–)-carvone applying the nitrosyl chloride method: a commercially important reaction (BAUER; GARBE; SURBURG, 2001).

Other widely investigated mechanisms for the allylic oxidation of olefins consist of the use of selenium dioxide (TRACHTENBERG; CARVER, 1970; SCHAEFER; HORVATH; KLEIN, 1968; TRACHTENBERG; TRACHTENBERG; NELSON; CARVER, 1970; WIBERG; NIELSEN, 1964), and many such terpene oxidation methods have been described in past years. In the case of limonene **(2)**, the reaction carried out in ethanol leads to the formation of limonen-4-ol **(31)** as the main product as well as limonen-10-ol **(32)** and carveol **(25)** in minor amounts (SAKUDA, 1969; THOMAS; BUCHER, 1970), while limonene-1,2-diol **(34)** was favored in the SeO_2-H_2O_2 system (WILSON; SHAW, 1973; SUMIMOTO; SUZUKI; KONDO, 1974). Mirtenal **(52)** may be prepared from α-pinene **(6)** using SeO_2-V_2O_5 with relatively high yields (LI, 2000a; ZHENG; LU, 1995), or using SeO_2 in ethanol (LIN; LI; DENG, 1990; LIU; DENG; CHENG, 2000), and the kinetics of this oxidation has been studied for the selenium dioxide-vanadium system (LI, 2000b). Selenium dioxide was also applied to induce the oxidation of β-pinene **(7)** (COXON; DANSTED; HARTSHORN, 1970; COXON; DANSTED; HARTSHORN, 1977; JOSHEL; PALKIN, 1942; STALLCUP; HAWKINS, 1941; STALLCUP; HAWKINS, 1942), camphene **(8)** (HIRSJARVI, 1956; HIRSJARVI; HIRSJARVI; KAILA, 1957; HIRSJARVI;

HIRSJARVI, 1965) and some sesquiterpenes (SATHE *et al.*, 1966). However, the possible formation of selenium and organoselenides in these kinds of reaction represent a problem to be considered, since selenium compounds are exceedingly toxic. This might be one of the reasons why such terpene oxidation methods are now in disuse.

The metal-catalyzed oxidation of terpenes has been extensively studied and might be an option for producing oxygenated derivatives. The palladium-catalyzed oxidation of limonene **(2)** and α- **(6)** and β-pinene **(7)** occurs mainly at the allylic sites of the molecule, and the main products are generally carvyl derivatives (GONÇALVES; BUENO; GUSEVSKAYA, 2006; GUSEVSKAYA; GONSALVES, 1997; GUSEVSKAYA; ROBLES-DUTENHEFNER; FERREIRA, 1998; EL FIRDOUSSI *et al*., 1998). In fact, the mechanism of such oxidations seems to be *via* the intermediate formation of π-allyl palladium complexes (GONÇALVES *et al*., 2005).

Allal *et al.* (2003) evaluated the influence of the catalyst and the reaction conditions in the oxidation of α-pinene **(6)**. The conclusion was that the system Cu/*t*-BuOOH/O_2/70°C promoted the formation of verbenone **(50)**, while Pd/H_2O_2/70°C yielded verbenol **(49)**. These systems were also studied in the oxidation of limonene **(2)**, 3-carene **(9)** and valencene **(58)**, and the best results were obtained when using Cu/*t*-BuOOH/O_2, which, in the case of valencene **(58)**, yielded nootkatone **(61)** with 100% conversion and 80% selectivity. The oxidation of α-pinene **(6)** catalyzed by other metal compounds, using H_2O_2 as the oxidizing agent has also been reported (MAKSIMCHUK *et al*., 2005, see also references cited). The same oxidizing agent and a metal complex biomimetic catalyst to metalloenzyme methane monooxigenase were used to oxidize limonene **(2)**, α- **(6)** and β-pinenes **(7)**. The main products obtained were, respectively, the ketones carvone **(26)**, verbenone **(50)** and pinocarvone **(54)** (CAOVILLA *et al*., 2008).

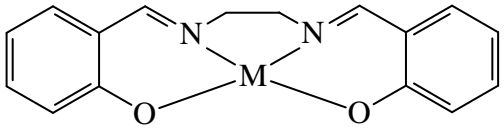


Figure 6. Basic structure of metal (salen) complexes.

The oxidation of monoterpenes using metal(salen) complexes (Figure 6) as catalysts has been widely described in recent years and it seem to replace the traditional techniques for the chemical reactions. These catalytic systems might be considered as cytochrome P450 analogs, since they involve oxometallic species (M=O) *via* a rebound mechanism such as the metalo-porphyrins (GOMES; ANTUNES, 1996b). When using iodosobenzene as the terminal oxidant and relative concentrations of limonbene:catalyst:iodosobenzene in a molar ratio of 1:0.03-0.05:1, the conversion of limonene **(2)** reached 50-60%, and the selectivities observed for *cis*- and *trans*-limonene-1,2-oxide **(33)** were 30% and 16.7%, respectively, 18.4% for carvone **(26)** and 10% for the two diastereoisomers of 1-*p*-menthen-9-al **(41)** (GOMES; ANTUNES, 1996a). Under the same conditions, a conversion of 50-60% was observed for α-pinene **(6)**, yielding, after 16h reaction, over 20% of a mixture of the corresponding epoxides **(56)** (55% selectivities) and between 2 to 6% of pinocamphone **(55)** and myrtenol **(51**. For β-pinene **(7)** the optimal conversion (55%) was obtained after 4 h reaction, with the maximum yield of myrtenal **(52)** isomers and epoxide **(57)** isomers varying from 6.5 to 23.2% and 2 to 4%, respectively (GOMES; ANTUNES, 1996b). In a recent study, Lima *et al*. (2006) evaluated the main reaction parameters that could affect the allylic oxidation or epoxidation of the metal(salen)-catalyzed oxidation of limonene **(2)**. The use of supercritical CO_2 instead of ordinary organic solvents was also investigated. In this system, the conversion was similar to that obtained in some organic solvents

but the higher selectivities towards the epoxide **(33)** formation, as observed for organic solvents, only occurred after 4 h reaction (LIMA *et al*., 2005).

Other cytochrome P450-biomimetic chemical systems (generally based on metaloporphyrins) capable of carrying out alkane hydroxylation and alkene epoxidation have also been reported (GROVES; SUBRAMANIAN, 1984; MOGHADAM *et al*., 2004). In the specific case of terpenes, Skrobot *et al*. (2003) showed the production of epoxides from these compounds. In an analogous work, the oxidation of monoterpenes by hydrogen peroxide catalyzed by porphyrins was also described (MARTINS *et al*., 1999; MARTINS *et al*., 2001). Other authors have made use of a photoexcitated porphyrin to oxidize limonene **(2)** and produce a mixture of carvone **(26)** and another unknown product (with a mass spectrum similar to that of verbenone **(50)**) in concentrations of up to 3.4 $g.L^{-1}$ and 6.0 $g.L^{-1}$, respectively. However, the oxidative degradation instability of the metaloporphyrins and the difficulty of recovering this expensive catalyst limit their practical application. In this case one possible solution might be the immobilization of the metaloporphyrins on solid supports (TRYTEK *et a*l., 2005).

Photooxidations via singlet oxygen employing dyes as photosensitizers have increasingly attracted the interest of organic chemists for industrial scale production of flavor compounds. This green chemical approach is one attractive alternative to the traditional chemical synthesis since it is a clean, traceless and sustainable technology, although the high energy demand of most artificial sources is one challenge that must be overcome (OELGEMÖLLER; JUNG; MATTAY, 2007). It has been employed in the production of ascaridole **(45)** from α-terpinene **(3)** (WOOTTON; FORTT; DE MELLO, 2002), the oxidation of α-thujene **(10)** (POHLMANN *et al*., 1997) and others. The photooxidation of citronellol **(11)** for the production of rose oxide **(43)** is the most distinguished example, a reaction currently performed industrially on a >100 tons per year scale by Symrise (OELGEMÖLLER; JUNG; MATTAY, 2007). This reaction begins with

the formation of two hydroperoxides in the presence of molecular oxygen, light and a photosensitizer (usually rose Bengal or methylene blue), which are than reduced with Na_2SO_3 to the corresponding regioisomers alcohols; only one of them is converted by acid cyclization to form an epimeric mixture of rose oxides (Figure 7). Due to is industrial importance, this photoreaction is currently used as a prototype for comparison studies, being used as a model for the investigation of the reaction parameters in a photomicroreactor (MEYER *et al*. 2007), for photoreactions under concentrated sunlight in the presence (OELGEMÖLLER *et al*., 2005; OELGEMÖLLER; JUNG; MATTAY, 2007) or absence of singlet oxygen (DINCALP; IÇLI, 2001) and to study the use of ionically polymer bound photosensitizer (GERDES *et al*., 2001).

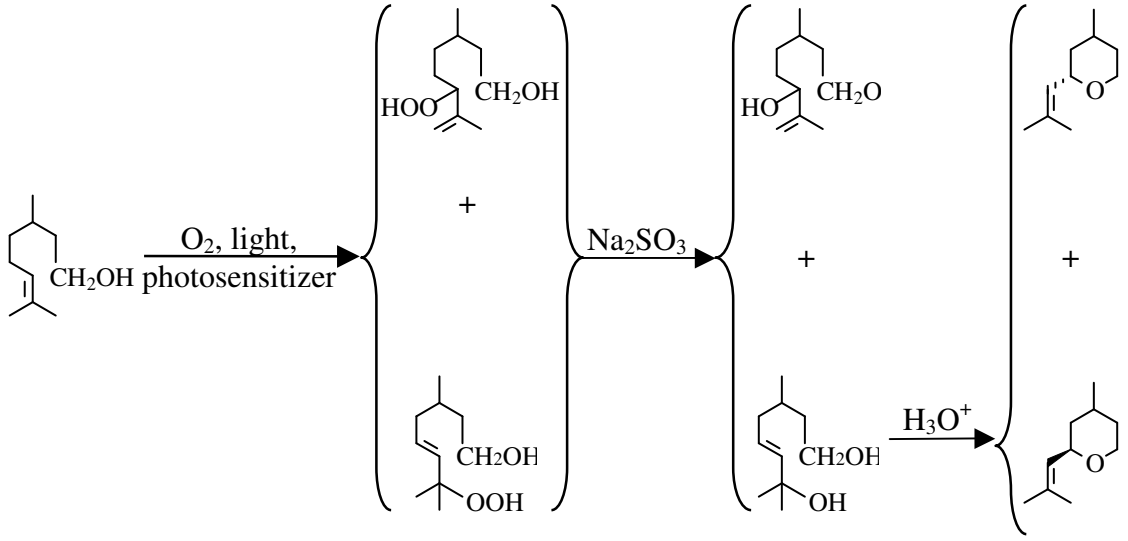


Figure 7. Photooxidation of citronellol for the production of rose oxide isomers (based on GERDES *et al.*, 2001).

For more detailed information on the chemical transformations of terpenes focused on the flavor industry, the reading of Swift (2004) and Monteiro and Veloso (2004) is recommended.

3. Biotransformation Processes

During recent years there has been increasing pressure on the industries to adapt their processes and products to recent global tendencies. Environmental concern has forced the development of cleaner processes, according to the 3R rule (Reduce, Reuse and Recycle), while the "dietetic revolution" imposes a growing demand for natural and, more recently, functional products containing the so-called bioactive compounds. In this context, biotransformation emerges as an attractive alternative for terpene oxidation since, as compared to the traditional chemical methods, they proceed under mild conditions, have an elevated regio and enantioselectivity, do not generate toxic wastes, and the products obtained can be labeled as "natural" (GIRI *et al*., 2001; JANSSENS *et al*., 1992; LEUENBERGER, 1990; SERRA; FUGANTI; BRENNA, 2005). In addition, the most significant strength of biotransformation processes is the ability to produce compounds not easily prepared by chemical methods.

Biotransformations can be briefly described as chemical reactions catalyzed by microorganisms or enzyme systems (KIESLICH, 1984) and are usually carried out with growing cultures, previously grown cells, immobilized cells, purified enzymes or multiphase systems (LEUENBERGER, 1990). According to De Carvalho and Da Fonseca (2006), 7% of the papers on terpene biotransformation published in the last ten years use purified enzymes as the biocatalyst, while plant cells, fungi, yeasts and bacteria account for 11%, 33%, 2% and 41%, respectively. In sequence, the (bio)oxidation of terpenes via biotransformation processes using isolated enzymes, whole plant cells and microorganisms will be considered in detail.

3.1. Use of Purified Enzymes

The use of purified enzymes in bioconversions may be advantageous or necessary in some cases, such as (i) when the membrane of the intact cell prevents appropriate substrate or product permeation, (ii) when there is posterior product degradation or undesirable side reactions involving other enzymatic systems, (iii) when the enzyme of interest is excreted by the cell and might be easily purified from the medium after biomass removal or (iv) when the enzyme of interest is commercially available. On the other hand, enzyme purification is often tedious, time consuming and expensive (LEUENBERGER, 1984).

The enzyme-generated reactive oxygen species process is a method that combines chemical oxidation and the enzymatic production of the oxidizing agent. This system can be applied to the biotechnological production of the aroma compound β-ionone **(66)** from β-carotene **(63)** by the use of enzymes, *eg.* lipoxygenase (WACHÉ; RATULD; BELIN, 2006; WU *et al*., 1999) and xanthine oxidase (BOSSER; BELIN, 1994). However, to be cleaved, the lipophilic substrate needs to be present in the aqueous phase where the enzymes work. An alternative to overcome this problem might be the use of micelles dispersed in water or solvent (WACHÉ; RATULD; BELIN, 2006).

An enzymatic system developed by Trytek and Fiedurek (2002) is apparently less sensitive to substrate concentration and temperature variation when compared to the microbial transformation methods. In this study, the optimum medium conditions for the conversion of limonene **(2)** to carvone **(26)** (apart from other side products) using glucose oxidase and horseradish peroxidase were pH 7.0, 1.5% substrate, 50°C and a reaction time of 16 to 24 h. This work was of great scientific value, since it described an original method for biotransforming a monoterpene using cell-free enzymes. However, the yield obtained was too low (<10 $mg.L^{-1}$) for

an industrial application, thus enzyme immobilization techniques might be considered for future similar studies.

Horseradish peroxidase was also studied in the enzymatic oxidation of citronellol **(11)**. This reaction occurs predominantly after double C–C linkage epoxidation reactions, followed by epoxide solvolysis (KAMIŃSKA *et al.*, 1989). Another peroxidase, present in the mycelium-free culture supernatant of the edible fungus *Lepista irina*, was able to degrade β-carotene **(63)** yielding flavor compounds. The degradation occurred most efficiently at 34°C with a pH optimum between 3.5 and 4, and the main volatile breakdown products formed were β-ionone **(66)**, β-cyclocitral **(67)**, dihydroactinidiolide **(68)** and 2-hydroxi-2,6,6-trimethylcyclohexanone (ZORN *et al*., 2003b).

Alcohol dehydrogenase can be used in the production of food additives, especially flavoring agents. One example is the oxidation of geraniol **(15)** to geranial **(16)** using horse liver alcohol dehydrogenase in biphasic mediums (LEGOY; KIM; THOMAS, 1985). In the same paper, different organic solvents and three co-factor regenerating methods were studied. Other monoterpenoid oxidations catalyzed by alcohol dehydrogenase recovered from plants have been described elsewhere (DAVIES *et al*., 1973; HATANAKA; SEKIYA; KAJIWARA, 1976).

The investigation of sesquiterpene biosynthesis in chicory led to the characterization of a cytochrome P450 hydroxylase which was shown to hydroxylate β-elemene **(59)** (DE KRAKER *et al*., 2001), and in a further study was shown to be able to hydroxylate a range of other sesquiterpenes exogenous to the plant, mainly yielding the respective isopropenyl or isopropylidene alcohols (DE KRAKER *et al*., 2003).

Many other terpene enzymatic transformations in cell free systems have been described, although they were basically focused on the isolation and characterization of microbial enzymes

(CADWALLADER; BRADDOCK; PARISH, 1992) or plant enzymes for the elucidation of the biosynthetic pathways involving volatile terpenoid formation in vegetables, especially monoterpenoids in *Mentha* leaves (KJONAAS; MARTINKUS-TAYLOR; CROTEAU, 1982; KJONAAS; VENKATACHALAM; CROTEAU, 1985; KARP *et al*., 1990) and norisoprenoids in quince (FLEISCHMANN; STUDER; WINTERHALTER, 2002), star fruit (FLEISCHMANN; WATANABE; WINTERHALTER, 2003) or nectarines (BALDERMANN; NAIM; FLEICHMANN, 2005).

3.2. Use of Integer Cells

According to Duetz, Van Beilen and Witholt (2001), there are four main reasons to use whole cells rather than purified enzymes: (i) apart from its simplicity and economy, the use of whole cells protects the enzyme from shear forces and might extend the enzyme activity half-life in a stirred bioreactor; (ii) the removal of an enzyme from a membrane environment often leads to complete or nearly complete loss of activity; (iii) cascades of enzymatic reactions may be too complicated to perform *in vitro* because of the number of enzymes, cofactors and substrates involved; (iv) the stoichiometric consumption of cofactors during the enzymatic reaction or chain of reactions may make the use of whole cells attractive. Besides, when using whole cells, the addition of cofactors is not required (CHATTERJEE, 2004).

3.2.1. Plant-Cultured Cells

Plant cell cultures exhibit a vast potential for the production of specific secondary metabolites and may be used to transform cheap and plentiful substances, such as industrial byproducts, into rare and expensive products (GIRI *et al*., 2001). Cytochrome P450 oxygenases from certain vegetable cells are known for their ability to oxidize monoterpenoids during their

biosynthesis. Hence, the use of these cells in the biotransformation of terpenes has been investigated in recent years (SUGA; HIRATA, 1990).

The biotransformation capacity of culture suspensions of *Achillea millefolium* was investigated using different monoterpenes and a mixture of farnesol **(62)** isomers. Except for geraniol **(15)**, the other substrates tested (borneol **(46)**, menthol **(40)**, thymol **(42)** and farnesols **(62)**) yielded less than 1 $mg.L^{-1}$ of products. The authors concluded that part of the substrates added and the biotransformation products were converted into and accumulated as the glycosylated forms (FIGUEIREDO *et al*., 1996). *Nicotiana tabacum* and *Catharanthus roseus* were investigated in the biotransformation of 3-carene **(9)** and α-pinene **(6)** (HIRATA *et al*., 1994), and cell culture suspensions of the last species were also tested in the conversion of (–)-piperitone **(37)**, which was regioselectively hydroxylated at the 4- and 6-positions (HAMADA *et al*., 1994). Cultured cells of *Caragana chamlagu* were able to convert α- **(65)** and β-ionone **(66)** into 3-oxo-α-ionone **(69)** and 5,6-oxi-β-ionone **(70)** with yields of 50% and 87%, respectively (SAKAMAKI *et al*., 2004). Further terpenic and non-terpenic substrates were tested in biotransformation assays using a culture suspension of *Peganum harmala* (ZHU; ASGHARI; LOCKWOOD, 2000).

Picea abies, from which the by-product turpentine is collected after the thermomechanical pulping process, has been widely studied in the biooxidation of terpenes using plant cell cultures. Their cell culture suspensions were tested in the biotransformation of α-pinene **(6)** (LINDMARK-HENRIKSSON *et al*., 2003), limonene **(2)** and β-pinene **(7)** (LINDMARK-HENRIKSSON *et al*., 2004) and the main products obtained were, respectively, *trans*-verbenol **(49)**, limonene-1,2-oxide **(33)** and *trans*-pinocarveol **(53**. α-Pinene **(6)** proved to be the fastest reacting substrate, but immobilization of the *Picea abies* cells decreased the transformation rate

without influencing the composition of the products or their absolute configuration (VANĚK *et al*., 2005). Immobilized *Solanum aviculare* and *Dioscorea deltoidea* cells were also applied to oxidize (–)-limonene **(2)**. However, in this case the attack was preferentially at position 6, yielding mainly *cis*- and *trans*-carveol **(25)** and carvone **(26)** (VANĚK *et al*., 1999).

Despite great academic interest, the insufficient enzymatic activity and low yields obtained limit the application of plant cell cultures in industrial processes.

3.2.2. Fungi and Yeasts

The use of microorganisms in monoterpene biotransformation is relatively recent, dating from the late 1950's and mid 1960's. Initially, the studies of microbial terpene bioconversion were based on the discovery of the metabolic pathways through which the substrates were metabolized. In fact, this mechanistic data are essential for the bioprocess engineering in case of bioflavors production. But, in the last years, the research in this field has been based on the discovery of novel flavor compound and on the optimization of the process conditions, although many paper still deals with the elucidation of terpene metabolism by microorganisms. The main microbial metabolic routes for limonene **(2)** (MARÓSTICA, PASTORE, 2007a; VAN DER WERF; SWARTS; DE BONT, 1999), α- **(6)** and β-pinenes **(7)** (BICAS *et al*., 2008b) and others (DEMYTTENAERE, 2001) have been well reviewed recently.

In pioneer studies, a soil pseudomonad was used for the microbial degradation of camphor **(46)** (BRADSHAW, 1959; BRADSHAW *et al*., 1959; CONRAD; DUBUS; GUNSALUS, 1961), limonene **(2)** (DHAVALIKAR; BHATTACHARYYA, 1966; DHAVALIKAR; RANGACHARI; BHATTACHARYYA, 1966), α- **(6)** and β-pinenes **(7)** (SHUKLA; BHATTACHARYYA, 1968; SHUKLA; MOHOLAY; BHATTACHARYYA,

1968), citronellol **(11)**, farnesol **(62)** and others (SEUBERT, 1960). The fungal-mediated oxidation of terpenes was described in the same period, after an *Aspergillus niger* capable of metabolizing α-pinene **(6)** to oxygenated products was selected amongst different molds (BHATTACHARYYA *et al*., 1960). Substrate concentrations of 0.6% ($v.v^{-1}$), an 8 h reaction time and a temperature range of 27-28 °C maximized the yields of the tree main metabolites (verbenol **(49)**, verbenone **(50)** and *trans*-sobrerol **(38)**) (PREMA ; BHATTACHARYYA, 1962a). Subsequently, the same *A. niger* strain was investigated in the conversion of other mono and sesquiterpenes (PREMA ; BHATTACHARYYA, 1962b).

Currently *A. niger* is one of the most extensively studied fungal species involved in monoterpene biotransformation. Some of the parameters involved in the transformation of α-pinene **(6)** to verbenone **(50)** by an *A. niger* isolated from soil underneath citrus trees, were optimized one-at-a-time. The optimal conditions were obtained when the microorganism was incubated for 6 h with 200 $mg.L^{-1}$ of substrate and 6 $g.L^{-1}$ of glucose in a sodium phosphate buffer at pH 7.0. Although the product formation increased, the yield remained low (328 $mg.L^{-1}$) (AGRAWAL; JOSEPH, 2000a). *A. niger* ATCC 9462 was investigated for the conversion of (–)-α-pinene **(6)**, (–)-β-pinene **(7)** and (+)-limonene **(2)**, but only the second compound was transformed by this strain. The best results, about 4% conversion of (–)-β-pinene **(7)** to α-terpineol **(30)**, were achieved when the substrate was supplemented in five subsequent additions as a 1:1 ethanol solution. Cell induction did not affect the reaction yield (TONIAZZO *et al*., 2005). Likewise, five monoterpene substrates, *i.e.* (+)- and (–)-limonene **(2)**, α- **(6)** and β-pinene **(7)** and camphor **(47)**, were used for the microbial production of aromas and fragrances by *A. niger* IOC-3913. The study was carried out in a liquid medium (with growing cells, pregrown cells and immobilized cells) or in a solid medium, with a substrate supply via the gas phase. (+)-

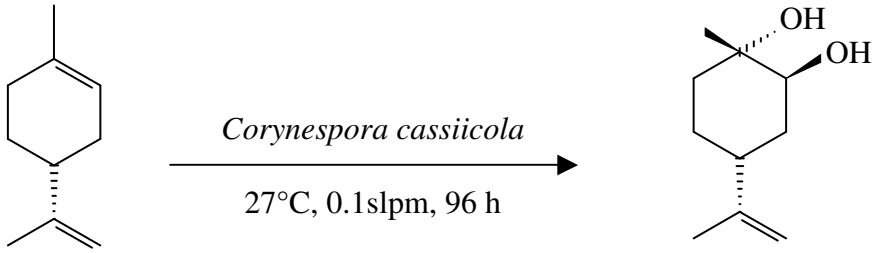
and (–)-limonene **(2)** were not metabolized by the strain tested, whilst verbenone **(50)** and α-terpineol **(30)** were the main products after the biotransformation of α- **(6)** and β-pinene **(7)**, respectively (ROZENBAUM *et al*., 2006). Another *A. niger* stain tested for the biotransformation of (+)- and (–)-limonene **(2)**, (+)- and (–)-α-pinene **(6)** and (–)-β-pinene **(7)** gave only satisfactory results for (–)-limonene **(2)**, yielding 18% of carveol **(25)** and 15% of dihydrocarveol **(39)** (DIVYASHREE; GEORGE; AGRAWAL, 2006). Further papers have described the biotransformation of limonene **(2)** to perillyl alcohol **(27)** (MENÉNDEZ *et al*., 2002) and the conversion of linalool **(18)** to furanoid **(19)** and pyranoid linalool **(20)** oxides mediated by *A. niger* (DEMYTTENAERE; WILLEMEN, 1998).

Larger terpenoid molecules have also been used in biocatalytic studies with *A. niger*. Mikami *et al.*(1981) selected an *A. niger* strain capable of transforming β-ionone **(66)** and β-methyl-ionone **(71)** into analogous tobacco-related aroma compounds. Another *A. niger* strain was found to be an efficient biocatalyst for a similar process, producing about 2.5 $g.L^{-1}$ hydroxyl and oxo derivatives from β-ionone **(66)** after 230 h cultivation (LARROCHE; CREULY; GROS, 1995). Later, the same strain was immobilized in calcium alginate beads due to the low aqueous solubility of the precursor, and the reaction carried out in a two-phase liquid system. The best yield, 3.5 $g.L^{-1}$, was obtained after 400 h reaction (GRIVEL; LARROCHE, 2001). The physicochemical parameters of that system were analyzed elsewhere (GRIVEL; LARROCHE; GROS, 1999). In their paper, very interesting for its originality, Krings *et al.* (2006) reported the screening of submerged microbial cultures able to oxifunctionilize the sesquiterpene α-farnesene **(60)**. One culture, identified as *A. niger*, exhibited the most versatile and attractive flavor profile. The oxidation of the sesquiterpenes valencene **(58)** and nootkatone **(61)** could also be performed by a soil-isolated *A. niger* as well as by other fungal strains (FURUSAWA *et al*., 2005).

In addition, *Aspergillus* sp., *i.e. A. cellulosae*, was capable of converting both enantiomers and the racemate of limonene **(2)** into limonene-*trans*-1,2-diol **(34)** as the main product (NOMA; YAMASAKI; ASAKAWA, 1992). In fact, some authors concluded that diols are common intermediates in the monoterpene metabolism of fungi (MUKHERJEE; KRAIDMAN; HILL, 1973).

Penicillium sp. is another fungal genera well documented in terpene biocatalysis. It was observed that the biotransformation of limonene **(2)** by *P. digitatum* occurred in the first instants of the log phase and that the bioconversion activity was expressively enhanced by the addition of substrate during the microbial growth (TAN; DAY; CADWALLADER, 1998). Other publications have described the biotransformation of limonene **(2)** to α-terpineol **(30)** using immobilized *P. digitatum* cells (TAN; DAY, 1998b) and have studied the effects of cosolvents in this conversion (ADAMS; DEMYTTENAERE; DE KIMPE, 2003; TAN; DAY, 1998a). According to other authors, the culture conditions (age of the culture, pH, glucose concentration and nitrogen source), substrate concentration, the amount of biomass, the pH of the buffer, the temperature and the incubation time taken for biotransformation of α-pinene **(6)** by a *Penicillium* sp., were found to be very critical for verbenone **(50)** formation. A 15-fold increase in product recovery was observed under the optimized conditions: 100 mL of a 0.05 M phosphate buffer pH 7.0 incubated at 30 °C for 6h with 20 mg of substrate and 200 mg of fungal biomass, which was harvested after 18 h-growth at 30 °C and pH 5.75 in potato dextrose agar, supplemented with 1% glucose and 0.025% yeast extract (AGRAWAL; JOSEPH, 2000b). Curiously, the biotransformation using spores of *P. italicum* (DEMYTTENAERE; DE POOTER, 1996) or *P. digitatum* ATCC 201167 (DEMYTTENAERE; DE POOTER, 1998) was feasible for, respectively, geraniol **(15)** and nerol **(12)** or citral **(14)** and nerol **(12)**, yielding 6-methyl-5-

hepten-2-one. The pathway involved in this kind of transformation was subsequently studied (WOLKEN; VAN DER WERF, 2001).



R-(+)-limonene
1,300 g

1*S*,2*S*,4*R*-limonene-1,2-diol
900 g

Figure 8. An economic way for preparing 1*S*,2*S*,4*R*-limonene-1,2-diol from *R*-(+)-limonene in a 100L bioreactor (ABRAHAM *et al*., 1985).

Amongst various different mono and sesquiterpenoids transformations mediated by fungi, such as *Corynespora cassiicola* and *Diplodia gossypina*, Abraham *et al*. (1985) described a well distinguished process of recovering good yields of 1*S*,2*S*,4*R*-limonene-1,2-diol **(34)** from *R*-(+)-limonene **(2)** with continuous substrate feeding in a 100 L bioreactor filled with 70 L of culture medium. When 1,300 g of substrate were used, 900 g of 1*S*,2*S*,4*R*-limonene-1,2-diol **(34)** and small amounts of the 1*R*,2*R*,*4R*-diasteroisomer were recovered after a 96 h-process, representing an economic way of preparing diols (Figure 8). The psychrotrophic *Mortierella minutissima* (TRYTEK; FIEDUREK, 2005) was also studied for the fungal conversion of limonene **(2)** into perillyl alcohol **(27)**/perillyc acid **(29)** and the best results, approximately 120 mg perillyl alcohol **(27)** per liter, were obtained after 120 h at 15 °C and pH 6.0. The authors concluded that the use of lower temperatures might reduce volatilization of the substrate and product, favoring the biotransformation process. Apparently, this is the only terpene biotransformation process

applying a psychrotrophic microorganism. In another manuscript, it was shown that *Cladosporium* sp. could transform limonene to α-terpineol **(30)** (KRAIDMAN; MUKHERJEE; HILL, 1969). The same transformation was feasible when an agroindustrial residue (cassava wastewater) was employed as an alternative culture medium for fungal cultivation, in this case a *Fusarium oxysporum* strain (MARÓSTICA JR.; PASTORE, 2007b). Using a similar technique, Maróstica Jr. and Pastore (2006) noticed that *Penicillium* sp. was able to produce *cis* and *trans* rose oxides **(43)** from citronellol **(11)**, like *Cystoderma carcharias* (ONKEN; BERGER, 1999a). The use of agroindustrial residues in bioprocesses seems to be a rising trend to overcome high manufacturing costs (PANDEY *et al*., 2000a) (see also cited references), including the production of flavors (CHRISTEN *et al*., 2000). Another alternative for the process optimization is the use of statistical methodology (e.g. Response Surface Methodology) to evaluate different parameters at the same time. This approach has been employed in the production of α-terpineol **(30)** (BICAS *et al*., 2008a) and other flavor compounds (MELO, PASTORE, MACEDO, 2005).

An interesting alternative to generate flavor compounds was via the fungal conversion of larger terpene molecules to volatile breakdown products. In this context, Zorn *et al.* (2003a) described an original method for screening microorganisms able to cleave β-carotene **(63)** to flavor compounds. From more than 50 filamentous fungi, ten bleached the zone surrounding the mycelium when grown in β-carotene-containing agar plates, suggesting the consumption of tetraterpene. Submerged cultures of four selected strains, *i.e. Ganoderma applanatum*, *Hypomyces odoratus*, *Kuehneromyces mutabilis*, and *Trametes suaveolens*, formed dihydroactinidiolide as the sole conversion product from β-carotene **(63)**, while other carotenoid-derived volatile metabolites, mainly β-ionone **(66)**, were detected in the mycelium-free culture supernatants from *Ischnoderma benzoinum*, *Marasmius scorodonius*, and *Trametes versicolor*

(ZORN *et al*., 2003a). A mixed culture formed by *Bacillus sp*. and *Geotrichum sp*. produced tobacco aroma compounds from lutein **(64)** after formation of the intermediate β-ionone **(66)**. The second microorganism was responsible for the production of β-ionone **(66)**, while the bacilli modified it to the aroma compounds 7,8-dihydro-β-ionone **(72)** and 7,8-dihydro-β-ionol **(73)** (MALDONADO-ROBLEDO *et al*., 2003; SANCHEZ-CONTRERAS; JIMENEZ; SANCHEZ, 2000). As already reported for *A. niger*, the filamentous fungus *Lasiodiplodia theobromae* ATCC 28570 may also metabolize the flavor compound β-ionone **(66)** to a complex mixture of metabolites reminding one of the tobacco flavor (KRASNOBAJEW; HELMLINGER, 1982). A similar hydroxylation of α- **(65)** and β-ionone **(66)** at positions 3 and 4, respectively, was performed by selected strains of the bacteria *Streptomyces*. It was demonstrated that the transformation of α-ionone **(65)** proceeded with both high regio- and stereoselectivity (LUTZ-WAHL *et al*., 1998). Other fungal species, such as *Armillariella mella* (DRACZYŃSKA *et al*., 1985) and *Botrytis cinerea* (ALEU; COLLADO, 2001; FAROOQ *et al*., 2002) were able to biotransform, respectively, α- **(6)**/β-pinenes **(7)** and a great variety of other terpenes.

Novel fungal strains are continuously being selected based on their ability to biotransform terpenes, and a promising alternative for screening potential fungi is the solid phase microextraction (SPME) technique to identify the biotransformation products, such as limonene-1,2-diol **(34)**, α-terpineol **(30)** and the isomers of rose oxide **(43)**, for both sporulated surface and submerged fungal cultures (DEMYTTENAERE; VAN BELLEGHEM; DE KIMPE, 2001; DEMYTTENAERE; VANOVERSCHELDE; DE KIMPE, 2004).

Interestingly, as far as we know, there are only a few descriptions of yeast-mediated terpene biotransformation process. The yeast *Candida tropicalis* MTCC 230 has shown its capacity to oxidize α-pinene **(6)** to α-terpineol **(30)** with an overall yield of 77% after 96 h at 30

°C, when 0.5 $g.L^{-1}$ of substrate was used. The product concentration remained stable up to 120 h of reaction time (CHATTERJEE; DE; BHATTACHARYYA, 1999). In a recent manuscript, Pinheiro and Marsaioli (2007) described the use of whole *Trichosporum cutaneum* cells in batch reactions to prepare oxiderivates of *cis*-jasmone **(74)**, *R*-(–)-carvone **(26)**, α- **(65)** and β-ionones **(66)** and *R*-(+)-limonene **(2)**. Other examples are the conversion of limonene **(2)**, α-pinene **(6)**, β-pinene **(7)** and some monoterpenoids by yeast or yeast-like fungi (VAN DYK; VAN RENSBURG; MOLELEKI, 1998; VAN DYK *et al*., 1998; VAN RENSBURG *et al*., 1997), and the modification of hop aroma terpenoids by ale and lager yeasts (KING; DICKINSON, 2003). In this context, further reports of terpene biotransformations by yeasts would be of great scientific value.

3.2.3. Bacteria

Although the microorganism-mediated conversion of terpene seems to proceed *via* cytochrome P450 monooxygenases (BERNHARDT, 2006; DUETZ et al., 2003; UNGER; SLIGAR; GUNSALUS, 1986), there are indications that the cytochrome P450 oxygenases of *A. niger* are not involved in the transformation of limonene **(2)** to perillyl alcohol **(27)** (MENÉNDEZ *et al*., 2002). Meanwhile it has also been observed that the bacteria *Rhodococcus erythropolis* DCL14 initiated the biotransformation of limonene **(2)** with an epoxidation at position 1,2, catalyzed by a limonene monooxygenase whose activity was not dependent on cytochrome P450 (VAN DER WERF; SWARTS; DE BONT, 1999). The first step to find adequate biocatalysts is the screening of those solvent-resistant microorganisms that can use the substrate as sole carbon source. This indicates the existence of a substrate-degrading metabolic pathway, which can possibly accumulate interesting intermediate products.

Similar to the first studies, various pseudomonads have been applied to the biotransformation of terpenes (TRUDGILL, 1986). Members of this bacterial genus have shown good resistance to solvents (INOUE; HORIKOSHI, 1989), have the metabolic flexibility to grow in a wide range of organic compounds as the sole carbon source and possess a wide variety of oxygenases and related enzymes for the activation and cleavage of terpene molecules (TRUDGILL, 1986). Yoo, Day and Cadwallader (2001) isolated a soil pseudomonad that could metabolize both α- **(6)** and β-pinenes **(7)**, resisting concentrations of up to 10% of these terpenes. The possible pathway for the degradation of α- **(6)** and β-pinenes **(7)** by this pseudomonad was later described (YOO; DAY, 2002). In earlier studies, some workers detected acid metabolites accumulated by *Pseudomonas* PX1 (GIBBON; PIRT, 1971) and *Pseudomonas putida* PIN11 (TUDROSZEN; KELLY; MILLIS, 1977) after the oxidation, followed by the ring cleavage of α-pinene **(6)**, suggesting a different pathway from that determined in the above study. Years later, it was demonstrated that *P. putida* GS1 could convert limonene **(2)** solely to perillic acid **(29)**, which remained stable in the culture medium (SPEELMANS; BIJLSMA; EGGINK, 1998). In sequence, Mars *et al*. (2001) concluded, after analyzing two *P. putida* strains (GS1 e F1) and one recombinant *E. coli* strain, that the enzymes involved in this biocatalysis belonged to the *p*-cymene **(4)** degradation pathway. Another *P. putida* strain, MTCC 1072, has shown the ability to metabolize limonene **(2)** producing perillyl alcohol **(27)** and sobrerol **(38)**, with yields of 36% and 44%, respectively (CHATTERJEE; BHATTACHARYYA, 2001). Divyashree, George and Agrawal (2006) described a *P. putida* isolate capable of biotransforming (+)- and (–)-limonene **(2)**, (+)- and (–)-α-pinene **(6)** and (–)-β-pinene **(7)**. The most important flavor compounds obtained in this study, i.e. verbenol **(49)**, dihydrocarveol acetate and verbenone **(50)** in yelds of

35%, 20% and 10%, respectively, resulted from the bioconversion of (+)-α-pinene **(6)**. The other monoterpenes were metabolized into another oxidated products.

Other members of this genus have also been applied in the oxidation of monoterpenes. The ability of *Pseudomonas gladioli* to utilize limonene **(2)** as the sole carbon source was first described by Cadwallader *et al*. (1989). The microorganism attacked the molecule at positions 7 and 8 to form perillic acid **(29)** and α-terpineol **(30)**, respectively. The enzyme responsible for the α-terpineol **(30)** formation, an α-terpineol dehydratase, was further isolated and characterized (CADWALLADER; BRADDOCK; PARISH, 1992). This enzyme converted stereoselectively and stereospecifically *R*-(+)-limonene **(2)** to *R*-(+)-α-terpineol **(30)** (BRADDOCK; CADWALLADER, 1995). A soil-isolated bacteria identified as *P. maltophilia*, was used to conduct the transformation of α-pinene **(6)** using resting cells or culture broth in a 30L-fermentor. The main natural products were identified as limonene **(2)**, borneol **(46)** and camphor **(47)**, while the acid fraction contained perillic acid **(29)** and 2-(4-methyl-3-cyclohexenylidene) propionic acid. Based on its O_2 uptake, it was demonstrated that this strain readily oxidized a diversity of monoterpenoids, e.g. β-pinene **(7)**, limonene **(2)**, α-phellandrene **(5)**, 1,8-cineole **(44)** and others (NARUSHIMA; OMORI; MINODA, 1982). *Pseudomonas fluorescens* NCIMB 11671, a microorganism capable of completely degrading α-pinene **(6)** and to use it as sole carbon and energy source, initiated the α-pinene **(6)** metabolism by a NADH-dependent double bond epoxidation, followed by two rings cleavage by an energy- and cofactor-independent α-pinene oxide **(56)** lyase to form two aldehydes (isonovalal **(21)** and novalal **(22)**). After their oxidation, the resulting acids (isonovalic acid **(23)** and novalic acid **(24)**) formed 3,4-dimethyl-pentanoic acid, which integrated the β-oxidation (BEST *et al*., 1987). A similar pathway was also evidenced for *Nocardia* sp. (GRIFFITHS *et al*., 1987a,b). Further reports suggest a different dynamic for

this pathway, explaining the formation of novalal **(22)** by isomerization of isonovalal **(21)** (LAROCHE; FONTANILLE; LARROCHE, 2003; LINARES *et al*., 2008; ZORN; NEUSER; BERGER, 2004) The study of this metabolic route led to the development of an optimized method for isonovalal **(21)** production from α-pinene oxide **(56)** by *Pseudomonas rhodesiae* CIP 10749 (FONTANILLE, P.; LARROCHE, 2003; FONTANILLE; LE FLÈCHE; LARROCHE, 2002). Mutants of the *P. fluorescens* wild strain unable to grow on α-pinene **(6)** and/or α-pinene oxide **(56)** were isolated after chemical mutagenesis, in order to explore alternative pathways and to serve as a basis for subsequent cloning studies. In this paper, the authors proposed possible alternative pathways for the metabolism of α-pinene **(6)** (COLOCOUSI; SAQUIB; LEAK, 1996). Recently, *P. rhodesiae* CIP 107491 and *P. fluorescens* NCIMB 11671 have been screened for their ability to grow and bioconvert different terpene sources in biphasic medium. The results indicated that *P. rhodesiae* is a specialist for the bioconversion of the pinene family (α- **(6)** and β-pinenes **(7)**) while *P. fluorescens* could also metabolize limonene **(2)** in two ways, the most profitable being the production of α-terpineol **(30)** in concentrations of ~11g.L^{-1}. Additionally, the cofactor-independent (bio)isomerization of β- **(7)** to α-pinene **(6)** was described for the first time (BICAS *et al*., 2008b).

Some *Pseudomonas* species were tested for the biodegradation of acyclic monoterpenoids, and special attention was given to the use of citronellol **(11)** and geraniol **(15)** by *P. citronellolis* (CANTWELL *et al*., 1978) One member of the latter species, *P. citronellolis* DSM 50332, showed the ability to anaerobically degrade some monoterpenoids (HARDER; PROBIAN, 1995). This degradation can occur using a pathway earlier described for *P. citronellolis* (SEUBERT, 1960).

Rhodococcus opacus PWD4 cells, which can use toluene as their sole carbon source, hydroxylated *R*-(+)-limonene **(2)** at position 6, forming enantiomerically pure *trans*-carveol **(25)**.

The maximal concentration of this product was obtained after 2.5 h and the final yield was 94-97%. The posterior conversion of (+)-*trans*-carveol **(25)** into (+)-carvone **(26)** by *Rhodococcus globerus* PWD8 illustrates that this strain might have a potential application in the industrial production of this ketone (Figure 9) (DUETZ *et al*., 2001). Similarly, *Rhodococcus erythropolis* DCL14 was able to transform (–)-*trans*-carveol **(25)** into (–)-carvone **(26)**. In this case the use of a biphasic system improved the bioconversion rate (TECELÃO; VAN KEULEN; DA FONSECA, 2001). It is worth noting that, differently from the nitrosyl chloride methodology (Figure 5), this procedure does not involve a modification of the optical rotation. Some reaction parameters involved in the biotransformation of geraniol **(15)** to geranic acid **(17)** by *Rhodococcus* sp. strain GR3 were studied by Chatterjee (2004), who reported that the reaction occurred optimally at 30°C and that the product concentration reached a maximum after 96 h and increased with increase in the geraniol **(15)** concentration up to 1.0% ($v.v^{-1}$). A patent application describes the preparation of hydroxylmethylated terpenes, more specifically perillyl alcohol **(27)**, using the biotransformative capacity of a variety of bacteria, including members of the genus *Rhodococcus* sp. (DUETZ; WITHOLT; JOURDAT, 2004).

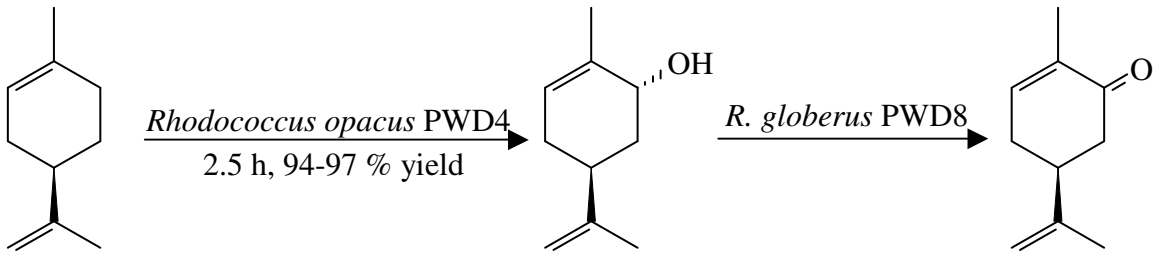


Figure 9. Biooxidation process of *R*-(+)-limonene to *trans*-carveol and carvone using *Rhodococcus* strains (DUETZ *et al*., 2001).

A *Xanthobacter* sp. isolated from river sediment, converted both enantiomers of limonene **(2)** into its 8,9-oxide **(35)** using cyclohexane as its sole carbon source, with the suggested involvement of a P450-dependent monooxygenase. The best results, 0.8 $g.L^{-1}$ of epoxide, were achieved using 12 mM of substrate concentration (VAN DER WERF; KEIJZER; VAN DER SCHAFT, 2000). The endobacterium *Serratia marcescens* has shown the capacity for two specific transformations of α-pinene **(6)**. In one of these biotransformations, the main product was *trans*-verbenol **(49)** together with minor amounts of verbenone **(50)** and *trans*-sobrerol **(38)**. Alterations in the culture conditions (use of another nitrogen source and the inclusion of glucose) changed the product profile, and in this case α-terpineol **(30)** was the major product formed (WRIGHT *et al*., 1986).

Some papers involving the *Bacillus* sp. metabolism of monoterpenes have also been published. In one of them, a strain isolated from pine trees, identified as *Bacillus pallidus* BR425, degraded α- **(6)** and β-pinene **(7)**, as well as limonene **(2)**. In the first case, significant amounts of pinocarveol **(53**, pinocarvone **(54)**, carveol **(25)**, carvone **(26)** and lesser amounts of myrtenol **(51**, myrtenal **(52)**, limonene **(2)** and β-pinene **(7)** were recovered. Carveol **(25)** was a common metabolite for all the monoterpenes tested, suggesting that this compound together with carvone **(26)**, are central growth intermediates in BR425 pinene metabolism (SAVITHIRY *et al*., 1998). In the same research field, Chang and Oriel (1994) isolated a thermophilic *Bacillus stearothermophilus* strain from orange peel that could use limonene **(2)** as the sole carbon source, converting it to perillyl alcohol **(27)** as the main product, and α-terpineol **(30)** and perillyl aldehyde **(28)** as minor products. The same compounds were obtained, although not in the same proportions, when a 9.6 Kb chromosomal fragment was cloned and expressed in the recombinant *Escherichia coli*, which could grow on limonene **(2)** as its sole carbon source. However, the level

of oxygenated monoterpenes recovered was considered insufficient for a possible industrial exploration of this process (CHANG; GAGE; ORIEL, 1995). In a following study, Savithiry, Cheong and Oriel (1997) separated the limonene **(2)** hydratation and methyl oxidation steps in the recombinant *E. coli* and noticed that a 3.8 Kb DNA fragment from the wild strain was responsible for growth on limonene **(2)** as sole carbon source. It was later demonstrated that the use of limonene **(2)** as the sole carbon source by the recombinant *E. coli* resulted from the expression of a single gene, which codified a new monoterpene oxidative enzyme producing carveol **(25)**, perillyl alcohol **(27)** and subsequently carvone **(26)** from limonene **(2)** (CHEONG; ORIEL, 2000).

3.3. Use of Unconventional Biocatalysts

Although the greater part of the biooxidation processes described in the literature is performed by microorganisms, different unusual biocatalysts have been tested for the conversion of terpenes. Marine microorganisms, such as the cyanobacteria *Synechococcus* sp. PCC 7942, which could hydroxylate both *S*-(–)-limonene **(2)** and its oxide **(33)** (HAMADA *et al*., 2003), and the unicellular microalgae *Dunaliella tertiolecta*, which reduced aldehydes to the corresponding primary alcohols (NOMA *et al*., 1992), are interesting examples. However, the most curious biocatalysts applied to terpene conversions are the superior organisms and animals.

Suggesting that the larvae of the cutworm *Spodoptera litura* possesses a high level of enzymatic activity against terpenoids, some authors have tested their biotransformation potential for limonene **(2)** (MIYAZAWA; WADA; KAMEOKA, 1998), α-terpinene **(3)** (MIYAZAWA; WADA; KAMEOKA, 1996), β-myrcene **(1)** (MIYAZAWA; MURATA, 2000), terpinen-4-ol **(36)** (MIYAZAWA; KUMAGAE, 2001), α-terpineol **(30)** (MIYAZAWA; OHSAWA, 2002),

menthol **(40)** (MIYAZAWA; KUMAGAE; KAMEOKA, 1999), camphor **(47)** (MIYAZAWA; MIYAMOTO, 2004), geraniol **(15)** (TAKECHI; MIYAZAWA, 2006) and others. The terpenic substrate (1-10 $mg.g^{-1}$, depending on the terpene tested) was mixed into the larvae's artificial diet and the products were analyzed (GC-MS) in the organic extract of their frass (insect excrement). In general, the unsaturated monoterpenes were hydroxylated at the allylic position. In this case, the terpene metabolism was similar to the terpene metabolism in mammals. Actually, there are several examples of terpenoid oxidations by mammals, although they have not received much attention (ISHIDA, 2005; SHIMADA; SHINDO; MIYAZAWA, 2002).

As may be observed there are interesting underexplored ways to biotransform terpenes, since every superior organism with a well developed enzymatic system, especially those involved in xeobiotic metabolism, have the potential to oxidize these compounds. Further research in this field could have great scientific value, especially if it discovers new compounds, unknown metabolites with unique structures, potential biocatalysts or original biotechniques.

4. Emergent Technology and Future Prospects

According to Leuenberger (1990), biotransformation might be a useful tool in organic chemistry, although some biotechnological developments are needed: optimization of the biocatalyst cultivation and biotransformation conditions (medium, temperature, agitation, pH *etc*.), strain improvement by classical methods or by genetic engineering, development of an appropriate production facility with an efficient product isolation procedure, process simplification to minimize the manufacturing costs and finally the scale up. Moreover, some techniques, which in combination with conventional methods could contribute to cost reductions and render further industrial biotransformation processes feasible and attractive, might be applied: the use of immobilized cells, improving biocatalyst stability and making a continuous

production process possible; the use of biphasic media, increasing the solubility of the substrate and avoiding its toxic effect towards the microorganism; and the use of recombinant DNA and protein engineering to improve the yields. In this context, many scientists are in search of genetically modified organisms for a more effective terpene oxidation process.

Unspecific genetic modifications through induced mutation (colchicine, ethyl methanesulphonate or ultraviolet radiation) of *Aspergillus* sp. and *Penicillium* sp., or protoplast fusion between members of these two genera were applied in order to improve verbenol **(49)** yields in the biotransformation of α-pinene **(6)** (AGRAWAL; DEEPIKA; JOSEPH, 1999; RAO; RAO; AGRAWAL, 2003). However, after the advent of DNA recombinant techniques, direct genetic approaches for increasing biotransformation rates and simplifying the process have been driving studies in this area.

Plant recombinant enzymes applied to hydroxylate *S*-limonene **(2)** (WÜST *et al*., 2001) and to cleave carotenoids producing apocarotenoid flavor compounds (SCHWARTZ; QIN; ZEEVAART, 2001) are already a reality. Additionally, some wild types and mutants of $P450_{cam}$ and $P450_{BM-3}$ have been investigated as a way to oxidize (+)-valencene **(58)** to (+)-nootkatone **(61)**. The latter presented higher activity although less selectivity when compared to $P450_{cam}$ (SOWDEN *et al*., 2005). *Pseudomonas putida* $P450_{cam}$, which is known to convert (+)-camphor **(47)** to 5-*exo*-hydroxycamphor **(48)**, was remodeled by designed mutagenesis, greatly enhancing activity for the oxidation of α-pinene **(6)** and *S*-limonene **(2)**. The authors suggested that this technique could give rise to novel fragrances and flavorings or new biologically active compounds (BELL; SOWDEN; WONG, 2001).

Although the approach of cloning and the expression of terpene biotransforming genes have already been performed in *E. coli* (CHANG; GAGE; ORIEL, 1995; CHEONG; ORIEL,

2000; SAVITHIRY; CHEONG; ORIEL, 1997), there are still only a few descriptions of the bio-oxidation of terpenes as carried out by cloned microorganisms, and the viability of such processes seems to be distant. One exception is the production of perillyl alcohol **(27)** from limonene **(2)** by *Pseudomonas putida,* expressing an alkene hydroxylase purified from *Mycobacterium* sp. HXN-1500. This process was performed in a 2-L bioreactor with a biphasic medium. After 75 h, the perillyl alcohol **(27)** accumulated in the organic phase reached 6.8 $g.L^{-1}$, equivalent to 2.3 $g.L^{-1}$ when calculated for the entire bioreactor contents (VAN BEILEN *et al*., 2005). As claimed by the authors, this is a promising technique for the industrial production of this alcohol.

However, it is worth noting that the success of innovative flavor biosynthesis does not depend exclusively on genetically improved biocatalysts, but also on process engineering, particularly when it comes to terpenoid flavor compounds (SCHRADER *et al*., 2004). As elsewhere stated (KASPERA, KRINGS, BERGER, 2006), genetic engineering is expected to be an universal solution in the future, however, until then, a careful selection of strain associated with appropriate bioprocess engineering will remain essential to obtain high yield processes.

5. Conclusions

The flavor and fragrance industries have grown constantly with the growth in the world economy. In parallel, the chemical oxidation of terpenes for flavor synthesis tends to be gradually replaced by biotechnological methods, and the rising quest for natural sources of aroma compounds is forcing an adaptation of the manufacturing methodology. Moreover, the biotechnological approach described in this review could be particularly useful for biorefineries or industries that can recover, based on a building-blocks concept, bioactive compounds from industry waste and by-products, resulting in a more sustainable flavor industry. Therefore, biotransformation processes, especially those applying filamentous fungi or bacteria, have arisen

as a promising alternative. However, the low transformation rates and high production costs are still obstructing their wide-scale adoption. To overcome these problems, genetic engineering technique seems to be a suitable choice, although such investigations are still in an embryonic stage. Therefore, more studies are essential to ensure the economical adoption of biotechnology for the production of flavor and fragrances.

The novel frontier in the field of food ingredients not only aims to provide good and economic technological applicability, but is also part of the quest for functional ingredients and nutraceuticals that is directing scientific and technological development in this area. Hence, flavor terpenes produced *via* biotransformation are completely adapted to the new market demand since, in addition to their natural nature, many of them have been proven to play an important biological role against certain types of cancer in *in vivo* studies.

CAPÍTULO 2

ISOLATION AND SCREENING OF *R*-(+)-LIMONENE-RESISTANT MICROORGANISMS

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1. Introduction

As already stated in the previous chapter, the main problems involved in monoterpene biotransformation processes are (i) the chemical instability of both precursor and product, (ii) the low solubility of the precursor, (iii) the high volatility of both precursor and product, (iv) the high cytotoxicity of both precursor and product and (v) the low transformation rates (KRINGS; BERGER, 1998). Therefore, a good screening of limonene-resistant strains is one of the most critical steps for choosing an adequate biocatalyst, since this might be a solution for the cytotoxicity and the low transformation rates.

The interest in solvent-tolerant microorganism screening is increasing in the last years. The isolation of microorganisms from this extreme environment will enable innovations in the field of fermentation and might bring several benefits to industries (HORIKOSHI, 1995). A solvent-resistant *Pseudomonas putida* strain for the limonene bioconversion has already been isolated from sludge (SPEELMANS; BIJLSMA; EGGINK, 1998).

Brazil is the major world producer of orange, presenting bulk amounts of *R*-(+)-limonene at a low price (MAZZARO, 2000). Consequently, the country has an enormous potential for

exploration of viable biotransformation process. This chapter describes the isolation and screening of microorganisms resistant to *R*-(+)-limonene and its use as sole carbon source, which is one of the main characteristics of a biotransforming agent. In the future, the selected strains might be evaluated for their biotransformation capacity.

2. Materials and methods

2.1. Samples

The *R*-(+)-limonene source used in this study was citrus terpene (94% *R*-(+)-limonene in GC, data not shown), a citric molasses oil gently supplied by Citrovita®.

Samples were collected in a citrus processing plant (Citrovita® – Araras, SP, Brazil), where it is believed to have strains more adapted to limonene-containing environment. From the eight samples, four of them were liquids ("yellow water" – YW –, "effluent" – E –, "entrance" – EN – and "exit" – EX – of the bagasse tank) while the other four were deteriorated semisolids fruits recovered from "orange bins" (OB – subdivided in five samples), "fruit washers" (FW – subdivided in four samples), "machine straps" (MS) and "plant floor" (PF – subdivided in six samples). Vegetable samples acquired in local market (oranges, lime, mint) were also used in this study.

For microorganism isolation, the inoculum was prepared as follows:

- Mint (M): small branch crushed under sterile conditions.
- Healthy orange (HO) (*Citrus sinensis* Osbeck), orange (*Citrus sinensis* Osbeck) with black dots (BD) and a rangpur lime (RL) (*Citrus limonia* Osbeck): 0.5cm x 0.5cm pieces of peel and pulp, obtained under sterile conditions.
- Liquid and semisolid samples: 2-3 loops with intercalated shaking.

2.2. Isolation and cultivation

Each inoculum described above was transferred to 250 mL Erlenmeyer flasks containing 50 mL of YM medium (1% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, pH not adjusted) and 50 μL (0.1%, v/v) of *R*-(+)-limonene, added to preselect limonene resistant strains. After 48 h or 7 days of cultivation in a rotary shaker at 30°C/150 rpm, a loop of each flask was transferred to Petri dishes containing YM medium (2% agar), following the streak plate method. The Petri dishes were incubated at 30°C until complete colonies growth, limited to 7 days. As each different colony appeared, these were transferred to 18 mL tubes with YM slant medium.

Fresh bacterial and yeasts colonies (24 h) were observed in microscopes after Gram coloration to confirm that they were actually isolated. In negative cases, the material was streaked in Petri dishes once more.

2.3. Selection of the strains

One loop of each isolated strain was transferred to a 50 mL Erlenmeyer flask containing 10 mL of sterile YM medium and 200 μL (2%, v/v) of *R*-(+)-limonene. The flasks were inoculated in a rotary shaker (30°C/150 rpm) for 48 h. After this period, a 100 μL sample of the culture broth was transferred to a Petri dish (YM medium) and homogenized with a Drigalski spatula. The culture growth was evaluated after 48 h at 30°C and all the strains that presented a satisfactory growth (>30 CFU) were considered resistant to 2% (v/v) of limonene.

In succession, all the strains considered resistant were evaluated in terms of the utilization of limonene as sole carbon source. Thus, one 100 μL aliquot of the culture broth from the last test was inoculated in a 50 mL Erlenmeyer flask containing 10 mL of mineral medium (in g/L:

$(NH_4)_2SO_4$ = 5.00; $(NH_4)_2HPO_4$ = 1.42; NaCl = 0.50; $MgSO_4.7H_2O$ = 0.40; $CaCl_2$ = 0.60; KCl = 2.15; $FeSO_4.7H_2O$ = 0.01; $ZnSO_4$ = 0.01; $CuSO_4$ = 0.01; pH not adjusted) and 100 µL (1%, v/v) of *R*-(+)-limonene. After 48 h incubation at 30°C and 150 rpm, a 100 µL sample of the culture medium was homogenized in a Petri dish (YM medium) as already described. All the colonies that presented a satisfactory growth (>30 CFU) after 48 h at 30°C were considered as possible users of *R*-(+)-limonene as sole carbon source.

3. Results and discussion

3.1. Isolated microorganisms

The first digit in Tables 1 and 2 shows the number of isolated microorganisms classes in this study. This evaluation is based on the morphology of the colony and their characteristics in microscope after Gram coloration.

Table 1. Number of microorganism classes isolated from different sources [a, b] after incubation (48h/30°C).

	YW	OB	MS	E	EN	EX	PF	FW	M	HO	BD		RL		Total
											pe	pu	pe	pu	
B G–	1/0/0	4/1/0	2/0/0	7/3/0	1/0/0	0/0/0	6/4/2	6/6/4	2/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	**29/14/6**
B G+	0/0/0	15/6/3	2/2/1	3/2/1	0/0/0	1/0/0	9/7/5	4/2/1	3/0/0	3/3/2	7/6/5	5/2/2	2/0/0	0/0/0	**54/30/20**
C G+	0/0/0	1/0/0	2/0/0	0/0/0	0/0/0	0/0/0	2/1/0	1/0/0	2/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	**8/1/0**
F	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	1/0/0	0/0/0	**1/0/0**
Y	2/0/0	6/1/0	1/1/0	0/0/0	1/1/0	1/1/1	5/4/0	2/2/2	0/0/0	0/0/0	2/0/0	0/0/0	0/0/0	0/0/0	**20/10/3**
Total	**3/0/0**	**26/8/3**	**7/3/1**	**10/5/1**	**2/1/0**	**2/1/1**	**22/16/7**	**13/10/7**	**7/0/0**	**3/3/2**	**9/6/5**	**5/2/2**	**3/0/0**	**0/0/0**	**112/55/29**

[a] YW: Yellow water; OB: orange bins; MS: machine straps; E: effluent; EN: entrance of bagasse tank; EX: exit of bagasse tank; PF: plant floor; FW: fruit washers; M: mint; HO: healthy orange; BD: orange with black dots; RL: rangpour lime; pe: peel; pu: pulp; B G–: Gram negative Bacillus; B G+: Gram positive Bacillus; C G+: Gram positive Coccus; F: fungus; Y: yeast.

[b] The first, second and third digits represents, respectively, the number of isolated microorganisms, the number of limonene resistant microorganism and the number of microorganisms that grow in medium with limonene as sole carbon source.

Table 2. Number of microorganism classes isolated from different sources [a, b] after incubation (7 days/30°C).

	YW	OB	MS	E	EN	EX	PF	FW	M	HO	BD		RL		Total
											pe	pu	pe	pu	
B G–	1/0/0	0/0/0	0/0/0	3/1/1	2/1/0	1/0/0	1/0/0	8/4/3	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	**15/6/4**
B G+	0/0/0	12/4/1	4/4/1	1/0/0	1/1/1	4/1/1	12/10/9	6/3/2	7/1/1	5/4/4	12/9/8	7/7/7	1/0/0	1/0/0	**73/44/35**
C G+	0/0/0	5/2/1	0/0/0	1/0/0	5/0/0	1/0/0	2/0/0	1/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	**15/2/1**
F	0/0/0	1/0/0	1/0/0	0/0/0	0/0/0	0/0/0	0/0/0	1/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	1/0/0	**4/0/0**
Y	1/0/0	4/1/0	1/1/0	0/0/0	1/1/0	1/1/0	4/4/1	3/3/0	1/1/0	1/1/0	1/1/0	0/0/0	0/0/0	0/0/0	**17/14/1**
Total	**2/0/0**	**22/7/2**	**6/5/1**	**5/1/1**	**9/3/1**	**7/2/1**	**19/14/10**	**19/10/5**	**8/2/1**	**6/5/4**	**13/10/8**	**7/7/7**	**1/0/0**	**2/0/0**	**126/66/41**

[a] YW: Yellow water; OB: orange bins; MS: machine straps; E: effluent; EN: entrance of bagasse tank; EX: exit of bagasse tank; PF: plant floor; FW: fruit washers; M: mint; HO: healthy orange; BD: orange with black dots; RL: rangpour lime; pe: peel; pu: pulp; B G–: Gram negative Bacillus; B G+: Gram positive Bacillus; C G+: Gram positive Coccus; F: fungus; Y: yeast.

[b] The first, second and third digits represents, respectively, the number of isolated microorganisms, the number of limonene resistant microorganism and the number of microorganisms that grow in medium with limonene as sole carbon source.

As may be observed, the majority of the microorganisms isolated were Gram positive Bacillus (127 strains), followed by a similar amount of both Gram negative Bacillus (44 strains) and yeasts (37 strains) and finally a lower number of Gram positive Cocci (23 strains). Interestingly, very few fungi strains could be isolated (5 strains), even though they grow very well in YM medium. This could be explained by the competition that could occur between the microorganisms, which might inhibit fungal growth. Other possibility is that the fungi, when

compared to bacteria and yeasts, are more susceptible to lower concentrations of *R*-(+)-limonene. Actually, limonene increases the fluidity of fungal membranes, which leads to a high unspecific membrane permeability and loss of membrane integrity (ONKEN; BERGER, 1999b).

The two incubation periods applied for the isolation, 48 h and 7 days, were chosen to favor the recovery of bacteria/yeasts and fungal strains, respectively. In general, the number of isolated microorganisms was higher for the samples incubated for 7 days. This longer period might favor stressed and sporulated microorganisms or those with a low growing rate. However, some microorganisms might enter in the death phase, which might render their isolation difficult. As expected, the number of fungi was higher for samples incubated for 7 days.

Pulp samples were collected in order to isolate some fruit endophytes. As may be observed in Table 1, the only class of possible endophyte isolated after 48h/30°C were Gram positive Bacillus from BD orange. On the other hand, after 7 days/30°C, BD orange resulted in seven Gram positive Bacillus and two yeasts, while rangpour lime gave one Gram poitive Bacillus and one fungi.

3.2. Limonene resistant microorganisms

The second digit in Tables 1 and 2 represents the number of limonene resistant microorganisms from those already isolated. It is worth noting that about one half of the isolated microorganisms resist limonene concentrations of up to 2%, except for the Gram positive Cocci, which appear to be more sensitive to limonene, and the fungi, that were not able to grow at high concentrations (≥2%) of limonene. In his study, Abraham (1994) reported that the taxonomic position of a strain is related to its enzymatic activity on terpenoid hydroxylation, but the

substrate toxicity could not explain the difference. Thus, substrate resistance does not guarantee a high biotransformation activity, but it is an essential characteristic to a biotransforming agent.

Generally, the minimum inhibitory concentration (MIC) of limonene against some bacteria and yeasts are lower than 2% (GRIFFIN *et al*., 1999), although some fungi, *e.g. Penicillium digitatum*, presents only a small reduction on its bioconversion activity in concentrations of 4-8% limonene (TAN; DAY; CADWALLADER, 1998) and some bacteria, *e.g. Pseudomonas* sp., might have no growth inhibition in concentrations of up to 10% α- or β-pinenes (YOO; DAY; CADWALLADER, 2001). Furthermore, monoterpene toxicity might depend on their droplet size in suspension, as observed for *Saccharomyces cerevisae* (URIBE; PEÑA, 1990). In biotransformation processes, the usual limonene concentration applied vary from 0.2 to 1% (CADWALLADER *et al*., 1989; DHAVALIKAR; BHATTACHARYYA, 1966; DHAVALIKAR; RANGACHARI; BHATTACHARYYA, 1966; MENÉNDEZ *et al*., 2002; NOMA, Y.; YAMASAKI, S.; ASAKAWA, 1992; TAN; DAY; CADWALLADER, 1998; VAN DER WERF; SWARTS; DE BONT, 1999), although 0.2% limonene is the optimum concentration for its biotransformation to perillyl alcohol and *p*-ment-1-ene-6,8-diol using *Pseudomonas putida* (CHATTERJEE; BHATTACHARYYA, 2001) and is toxic to *Bacillus stearothermophilus* (CHANG; ORIEL, 1994).

The methodology considered in this microorganism screening was similar to that applied by Chatterjee (2004), who selected a 0.3% geraniol resistant soil bacteria for biotransformation trials. However, the solid phase microextraction (SPME) technique may also be useful for fungal screening (DEMYTTENAERE; VAN BELLEGHEM; DE KIMPE, 2001). In this study, 121

microorganisms were selected with higher MIC values, which makes them greatly attractive to biotechnological purposes.

3.3. Use of limonene as sole carbon source

From the total of 238 isolated strains, 70 grew well in medium containing limonene as sole carbon source. The great majority of these microorganisms were Gram positive Bacillus, followed by a small number of Gram negative Bacillus and yeasts, and only one Gram positive Cocci. As there were no limonene resistant fungi, none of them were tested for utilization of limonene as sole carbon source.

Proportionally, the recovery of strains able to use limonene as sole carbon source is favored by a 7 day-incubation and the most adapted microorganisms appear to be the Gram positive followed by Gram negative Bacillus. This is in accordance with the bacterial biotransformation processes already published, as great part of them refers to *Pseudomonas* sp. (Gram negative Bacillus) (CADWALLADER *et al*., 1989; CHATTERJEE; BHATTACHARYYA, 2001; DHAVALIKAR; BHATTACHARYYA, 1966; DHAVALIKAR; RANGACHARI; BHATTACHARYYA, 1966; MARS *et al*., 2001; SPEELMANS; BIJLSMA; EGGINK, 1998; YOO; DAY; CADWALLADER, 2001) and *Bacillus* sp. (Gram positive Bacillus) limonene conversions (CHANG; ORIEL, 1994; CHANG; GAGE; ORIEL, 1995; GURAJEYALAKSHMI; ORIEL, 1989; NATARAJAN; LU; ORIEL, 1994). Even though a larger part of the isolated yeasts resists high limonene concentration, only a few (four for 48 h and one for 7 days) develop well in medium with this monoterpene as sole carbon source. This lower limonene metabolizing capacity might explain the few work published covering the yeasts-mediated biotransformation of limonene (VAN DYK; VAN RENSBURG; MOLELEKI, 1998;

VAN RENSBURG *et al*., 1997). The only isolated Gram positive Coccus is of importance due to its biotransforming potential. As far as we know, there is no limonene biotransformation study described in the literature using this class of bacteria.

Biotransformation processes with all the selected microorganisms were investigated in our laboratory in order to select the aroma-productive strains. None of the strains tested has shown an accumulation of intermediate metabolites in levels that justified further optimization studies. Therefore, experiments are underway to evaluate the possible pathways through wich these microorganisms degrade the limonene.

CAPÍTULO 3

OTIMIZAÇÃO DA PRODUÇÃO DE *R*-(+)-α-TERPINEOL PELA BIOTRANSFORMAÇÃO DO *R*-(+)-LIMONENO

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1. Introdução

Esse estudo foi conduzido com um microorganismo do Laboratório de Bioaromas já conhecidamente capaz de produzir lipase alcalina (PRAZERES, CRUZ, PASTORE, 2006) e *R*-(+)-α-terpineol a partir do *R*-(+)-limoneno (MARÓSTICA JR., PASTORE, 2007b). A linhagem escolhida, *Fusarium oxysporum* 152b, isolada de amostras de solo do nordeste brasileiro (PRAZERES, 2006) mostrou-se capaz de biotransformar o do *R*-(+)-limoneno contido no óleo da casca de laranja e a produção máxima foi de cerca de 400 mg de *R*-(+)-α-terpineol por litro de meio de cultura (MARÓSTICA JR., PASTORE, 2007b). Outros estudos relatam ainda a biotransformação do limoneno para o α-terpineol (principal produto) por linhagens de *Cladosporium* sp. (KRAIDMAN; MUKHERJEE; HILL, 1969), *Pseudomonas gladioli* (CADWALLADER; BRADDOCK; PARISH, 1992; CADWALLADER *et al.*, 1989) e *Penicillium digitatum* (ADAMS; DEMYTTENAERE; DE KIMPE 2003; TAN; DAY, 1998a,b; TAN; DAY; CADWALLADER, 1998). O α-terpineol é um álcool estável comumente produzido pele síntese química a partir do α-pineno ou da terebentina. É um produto comercial importante

tipicamente empregado em produtos de higiene, cosméticos e preparações de aromas (BAUER, GARBE, SURBURG, 2001). Portanto, para se tornar competitiva, a produção biotecnológica desse composto deve gerar rendimentos bastante satisfatórios, o que representa um mínimo de 1 $g.L^{-1}$.

O objetivo foi então dar continuidade a esse estudo otimizando as condições do processo e entender como os principais parâmetros interferem na quantidade de *R*-(+)-α-terpineol produzido empregando a metodologia de Plackett-Burman para a seleção e o Delineamento Composto Central Rotacional (DCCR) para a otimização das variáveis. Essas ferramentas estatísticas permitem, ao final do estudo, avaliar o efeito das variáveis estudadas e determinar as condições que maximizem a produção do composto de interesse. Otimizações da produção de compostos de aroma utilizando a Metodologia de Superfície de Resposta já foram publicadas (ÇELIK, BAYRAKTAR, MEHMETOĞLU, 2004; MELO, PASTORE, MACEDO, 2005). No entanto, esta técnica nunca foi descrita para a obtenção de monoterpenóides aromáticos, especialmente a formação de *R*-(+)-α-terpineol a partir do *R*-(+)-limoneno, apesar de que métodos clássicos foram relatados para a biotransformação de *R*-(+)-limoneno a *R*-(+)-α-terpineol pelo fungo *Penicillium digitatum* NRRL 1202 (TAN, DAY, CADWALLADER, 1998), assim como a biotransformação do limoneno a carvona pela preparação enzimática contendo glicose oxidase e peroxidase (TRYTEK, FIEDUREK, 2002) e a produção de verbenona a partir do α-pineno pelo fungo *Penicillium* sp. (AGRAWAL, JOSEPH, 2000b).

Este capítulo descreve a otimização de dez variáveis do processo de biotransformação do *R*-(+)-limoneno a *R*-(+)-α-terpineol pela linhagem *Fusarium oxysporum* 152b empregando uma matrix Plackett-Burman (PB-16) para a seleção de variáveis seguida por um DCCR.

2. Material e métodos

2.1. Reagentes

R-(+)-limoneno (Merck, >94% pureza), α-terpineol (Aldrich, 90% pureza) e *R*-(+)-α-terpineol (Fluka, ~99% pureza) foram mantidos sob refrigeração (4°C). O Biossurfactante de *Bacillus subtilis* foi produzido no Laboratório de bioaromas seguindo metodologia padrão (BARROS, 2007).

2.2. Inóculo

Uma placa de Petri contendo uma cultura de *F. oxysporum* 152b de 48 h foi dividida em três partes iguais e distribuída em três frascos cônicos de 500 mL contendo cada 200mL de meio YM (10 $g.L^{-1}$ glicose, 5 $g.L^{-1}$ peptona, 3 $g.L^{-1}$ extrato de levedura, 3 $g.L^{-1}$ extrato de malte, pH 6,7). O material foi homogeneizado em condições estéreis com um Ultra-Turrax® T18 até completa ruptura do material sólido. Apos inoculação por 72 h a 30 °C e 150 rpm a massa celular foi concentrada por filtração a vácuo em um funil de Buchner com papel de filtro Whatman n° 1. A separação foi interrompida quando o retentado atingiu 45% da massa inicial, resultando em uma contagem final de $1,6\pm0.2\cdot10^{7}$ $UFC.mL^{-1}$.

2.3. Experimentos de otimização

Devido à limitação das informações a respeito da influência dos principais parâmetros da biotransformação do *R*-(+)-limoneno a *R*-(+)-α-terpineol por *Fusarium oxysporum* 152b, um estudo extenso foi realizado a fim de definir os efeitos da composição do meio, das condições de

cultivo, da concentração de substrato e da relação inóculo/meio de cultivo na produção de *R*-(+)-α-terpineol (área do pico de *R*-(+)-α-terpineol em CG). Assim, a estratégia foi executar uma seleção de dez variáveis empregando uma matriz Plackett-Burman (RODRIGUES, IEMMA, 2005) com 16 experimentos (PB-16) e cinco pontos centrais para determinar os principais parâmetros (Tabelas 3 e 4). Um Delineamento Composto Central Rotacional (DCCR) com os parâmetros selecionados foi realizado na seqüência para definir as condições ótimas para o processo (Tabela 5 e 6). Os pontos centrais para a seleção de variáveis foram feitos com meio YM contendo 1,0 % (v/m) de substrato e uma relação inóculo/meio de cultivo de 0,5 % (m/m), incubados a 30 °C e 150 rpm (adaptado de Maróstica Jr e Pastore (2007b)), alem de uma concentração de biossurfactante de *Bacillus subtilis* LB5a equivalente a 1CMC (Concentração Micelar Critica, nesse caso 11mg/L). Os pontos centrais e amplitudes dos parâmetros do DCCR (Tabela 6) foram escolhidos baseados nos resultados preliminares (PB-16).

Tabela 3. Variáveis e níveis avaliados no planejamento de seleção.

Variáveis	Níveis		
	−1	0	+1
Composição do meio			
Glicose ($g.L^{-1}$)	0	10	20
Peptona ($g.L^{-1}$)	0	5	10
Extrato de levedura ($g.L^{-1}$)	0	3	6
Extrato de malte ($g.L^{-1}$)	0	3	6
pH	5,2	6,7	8,2
Biosurfactante ($mg.L^{-1}$)	0	10	20
Substrato			
Limoneno (%, v/m)	0,5	1,0	1,5
Condições de cultivo			
Temperatura (°C)	20	30	40
Agitação (rpm)	0	150	300
Inóculo			
Relação inóculo/meio (m/m)	0,25	0,50	0,75

Tabela 4. Matriz Plackett-Burman (PB-16) e a área de *R*-(+)-α-terpineol em CG-FID após 24, 48, 72 e 96 h de biotransformação [a, b].

	Gli	Pep	ExM	ExL	L	pH	S	T	A	I/M	área α-T ($x10^6$)			
											24 h	48 h	72 h	96 h
1	+1	−1	−1	−1	+1	−1	−1	+1	+1	−1	0,16	0,15	0,16	0,51
2	+1	+1	−1	−1	−1	+1	−1	−1	+1	+1	1,09	3,58	12,25	15,82
3	+1	+1	+1	−1	−1	−1	+1	−1	−1	+1	0,76	1,66	2,50	2,62
4	+1	+1	+1	+1	−1	−1	−1	+1	−1	−1	0,12	0,17	0,18	0,14
5	−1	+1	+1	+1	+1	−1	−1	−1	+1	−1	0,13	0,33	2,77	5,22
6	+1	−1	+1	+1	+1	+1	−1	−1	−1	+1	0,26	0,37	0,47	0,51
7	−1	+1	−1	+1	+1	+1	+1	−1	−1	−1	0,15	0,15	0,20	0,28
8	+1	−1	+1	−1	+1	+1	+1	+1	−1	−1	0,09	0,14	0,14	0,13
9	+1	+1	−1	+1	−1	+1	+1	+1	+1	−1	0,13	0,45	0,18	0,25
10	−1	+1	+1	−1	+1	−1	+1	+1	+1	+1	0,18	0,21	0,20	0,31
11	−1	−1	+1	+1	−1	+1	−1	+1	+1	+1	0,16	0,22	0,22	0,24
12	+1	−1	−1	+1	+1	−1	+1	−1	+1	+1	0,27	0,89	3,05	5,81
13	−1	+1	−1	−1	+1	+1	−1	+1	−1	+1	0,13	0,16	0,13	0,16
14	−1	−1	+1	−1	−1	+1	+1	−1	+1	−1	0,40	6,31	26,94	21,93
15	−1	−1	−1	+1	−1	−1	+1	+1	−1	+1	0,11	0,11	0,12	0,13
16	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	0,78	1,63	3,19	4,45
17	0	0	0	0	0	0	0	0	0	0	0,41	1,38	6,20	10,78
18	0	0	0	0	0	0	0	0	0	0	0,35	1,85	5,81	6,39
19	0	0	0	0	0	0	0	0	0	0	0,48	1,40	5,61	8,91
20	0	0	0	0	0	0	0	0	0	0	0,58	1,16	5,70	8,17
21	0	0	0	0	0	0	0	0	0	0	0,35	1,64	6,61	8,30

[a] Gli = glicose; Pep = peptona; ExM = extrato de malte; ExL = extrato de levedura; L = limoneno; S = biossurfactante; T = temperatura; A = agitação; I/M = relação inóculo/meio (m/m); área α-T = área do pico de *R*-(+)-α-terpineol. [b] Os níveis de cada variável são descritos na Tabela 3.

Tabela 5. Variáveis e níveis avaliados no DCCR.

Variáveis	níveis				
	−2	−1	0	+1	+2
Condições de cultivo					
Temperatura (°C)	10	15	20	25	30
Agitação (rpm)	0	90	180	270	360
Substrato					
Limoneno (%)	0,1	0,3	0,5	0,7	0,9
Co-substrato					
Biossurfactante ($mg.L^{-1}$)	0	125	250	375	500

Tabela 6. Matriz DCCR 2^4 e a área de *R*-(+)-α-terpineol em GC-FID apos 72 h e 96 h de biotransformação [a, b].

	T	A	L	S	area α-T ($x10^6$)	
					72 h	96 h
1	−1	−1	−1	−1	0,81	2,23
2	+1	−1	−1	−1	19,14	18,36
3	−1	+1	−1	−1	1,21	16,27
4	+1	+1	−1	−1	21,17	22,37
5	−1	−1	+1	−1	0,68	1,95
6	+1	−1	+1	−1	7,63	13,30
7	−1	+1	+1	−1	0,19	0,47
8	+1	+1	+1	−1	18,17	16,66
9	−1	−1	−1	+1	0,88	9,51
10	+1	−1	−1	+1	16,60	18,75
11	−1	+1	−1	+1	10,52	20,37
12	+1	+1	−1	+1	20,38	19,65
13	−1	−1	+1	+1	0,37	2,58
14	+1	−1	+1	+1	9,92	11,76
15	−1	+1	+1	+1	0,20	0,19
16	+1	+1	+1	+1	15,27	18,53
17	−2	0	0	0	0,18	0,19
18	+2	0	0	0	17,68	18,55
19	0	−2	0	0	2,35	4,31
20	0	+2	0	0	19,61	16,58
21	0	0	−2	0	0,20	7,89
22	0	0	+2	0	9,93	13,00
23	0	0	0	−2	15,33	18,76
24	0	0	0	+2	19,98	19,86
25	0	0	0	0	16,63	21,75
26	0	0	0	0	16,17	18,77
27	0	0	0	0	18,76	19,53
28	0	0	0	0	18,70	22,54
29	0	0	0	0	17,78	19,03
30	0	0	0	0	18,25	18,11

[a] Os níveis de cada variável são descritos na Tabela 6. [b] T = temperatura; A = agitação; L = limoneno; S = biossurfactante; área α-T = área do pico de *R*-(+)-α-terpineol.

2.4. Procedimento de biotransformação

A massa celular concentrada obtida no item 2.2 foi distribuída em frascos estéreis de 100mL com tampa rosqueável. Em seguida o meio de cultivo (pH ajustado antes da esterilização) e o substrato foram adicionados para totalizar uma massa final de 15 g. A proporção de cada componente variou de acordo com cada planejamento experimental como mostram as Tabelas 3 e 5. Cada frasco foi incubado em um agitador em suas respectivas condições (Tabelas 3 e 5).

Com o objetivo de definir a cinética de transformação, quatro planejamentos foram efetuados em paralelo, um para cada tempo de processo: 24 h, 48 h, 72 h e 96 h. Como os melhores resultados foram sempre obtidos por volta de 72 h a 96 h, optou-se por realizar dois DCCRs, um para 72 h de reação e outro para 96 h.

2.5. Quantificação e identificação dos compostos voláteis

Os compostos voláteis foram analisados pela metodologia de microextração em fase sólida (SPME). O aparato empregado (Supelco) consiste de uma fibra de sílica fundida de 10mm de comprimento recoberto com uma camada de 75 μm de CA*R*-PDMS. Antes das análises, a fibra foi precondicionada no injetor do cromatógrafo de acordo com as instruções do fabricante.

Os compostos de aroma foram extraídos de frascos de 100 mL com septo de PTFE contendo 50 μL do meio de fermentação diluído em 50mL de solução saturada de NaCl (360 $g.L^{-1}$). O sistema foi mantido por dez minutos a 40 °C em agitação para permitir o equilíbrio de fases. A fibra foi então exposta ao headspace por 30min a 40°C (WAGNER *et al*., 2007).

As análises foram feitas em um cromatógrafo gasoso Varian 3800 com detector por ionização de chama (CG-DIC). A dessorção ocorreu no injetor do equipamento por 1 min a 280 °C com a válvula de purga fechada (modo *splitless*). Os compostos voláteis foram separados em

uma coluna capilar DB-Wax de sílica fundida com 30 m x 0,25 mm i.d. x 0,5 µm espessura de filme (J&W Scientific). O programa de temperatura empregado foi isotérmico por 2 min a 50 °C, em seguida aumentou ate 200 °C a 5 °C min^{-1} onde permaneceu 5 min. Hélio foi o gás de arraste em uma vazão de 1,2 $mL.min^{-1}$ e a temperatura do detector foi de 250°C. Após a dessorção, a fibra foi mantida exposta por 10 min no injetor com intuito de eliminar a possibilidade de *carry-over* de analitos entre as amostras.

A quantificação foi feita por curva de calibração externa de α-terpineol, obtida pela distribuição de concentrações opostas de *R*-(+)-limoneno (4,23; 3,17; 2,11; 1,06 e 0,10 g por litro de meio) e α-terpineol (0,10; 1,12; 2,24; 3,36 e 4,20 g por litro de meio) em um frasco de 100 mL com tapa rosqueável contendo 3,75 g de inóculo e 11,25 g de água destilada (pontos centrais do DCCR), em duplicata. A identificação enantiomérica foi realizada comparando-se os padrões *R*-(+)-α-terpineol, α-terpineol (poder rotativo negativo, majoritariamente isômero S) e a amostra com relação ao tempo de retenção e *spiking*. Para tal, uma coluna capilar quiral Beta Dex™ 120 de sílica fundida (Supelco; 60 m, 0,25 mm i.d., 0,25 µm espessura de filme) com um programa de temperatura isotérmico a 140°C por 20 min, posteriormente elevado a 210°C a 20ºC.min^{-1} onde foi mantido por 5 min. As demais condições são as mesmas apresentadas acima.

Para a identificação dos produtos voláteis empregou-se um cromatógrafo gasoso Shimadzu 17A com coluna capilar DB-5 (J&W Scientific; 30 m x 0.25 mm i.d. x 0.25 μm espessura de filme) acoplado a um espectro de massas Shimadzu QP-5000 (CG-EM). O programa de temperatura foi de 60 °C elevada a 210 °C a 3 °C.min^{-1} a qual foi mantida por 5 min. O gás de arraste foi hélio a uma vazão de 1,0 $mL.min^{-1}$. A interface CG/EM foi mantida a 240 °C. O espectro de massas trabalhou em modo de impacto de elétron com uma energia de 70 eV e voltagem de 1,4 kV a 0,5 $scan.s^{-1}$ em uma faixa de *m/z* de 35-350.

2.6. Análise dos resultados

Os resultados foram analisados pelo *software* STATISTICA® 5.5 A. O nível de significância de 10 % (p<0,1) foi considerado para o planejamento de seleção de variáveis e 5 % (p<0,05) para o DCCR.

3. Resultados e discussão

3.1. Seleção das variáveis

Os pontos centrais para o planejamento de seleção foram escolhidos baseados nas condições usualmente utilizadas nessa biotransformação. Uma triplicata dos pontos centrais mostrou que antes de ocorrer a biotransformação (0 h) há uma pequena quantidade de *R*-(+)-α-terpineol (áreas de 0,7 a $0{,}9 \cdot 10^6$) no meio, provavelmente presentes como contaminante do substrato. Um experimento controle, sem inóculo, realizado nas mesmas condições do ponto central, demonstrou que a quantidade de *R*-(+)-α-terpineol no meio permanece praticamente estável na ausência do catalisador, apresentando somente um ligeiro aumento entre 0 h e 48 h (de 0,8 a $1{,}1 \cdot 10^6$), sem posterior aumento significativo ate 96 h, o que indica que o *R*-(+)-α-terpineol produzido nesse processo não provem da autoxidação do *R*-(+)-limoneno (dados não apresentados).

A Tabela 4 mostra que, com poucas exceções, a área de *R*-(+)-α-terpineol aumenta com o decorrer da reação, atingindo um valor máximo entre 72 h e 96 h, um perfil condizente com os resultados prévios (MARÓSTICA JR., PASTORE, 2007b). No entanto, o aumento observado entre 72 h e 96 h pode não ser suficiente para justificar um processo mais longo. Por esse motivo, tempos superiores a 96 h não foram avaliados no DCCR, pois a produtividade do processo diminuiria. A análise estatística dos resultados é apresentada na Tabela 7.

Tabela 7. Estimativa dos efeitos dos parâmetros analisados apos 72 h e 96 h de biotransformação [a, b].

Fator	Tempo	Efeito ($x10^6$)	EP ($x10^6$)	t(10)	p-valor
Media	**72 h**	**3,93**	**1,00**	**3,92**	**0,0029**
	96 h	**4,81**	**1,06**	**4,55**	**0,0011**
Gli	72 h	-1,85	2,30	-0,81	0,4386
	96 h	-0,87	2,42	-0,36	0,7277
Pep	72 h	-1,98	2,30	-0,86	0,4088
	96 h	-1,11	2,42	-0,46	0,6556
ExM	72 h	1,77	2,30	0,77	0,4599
	96 h	0,46	2,42	0,19	0,8521
ExL	**72 h**	**-4,79**	**2,30**	**-2,08**	**0,0637**
	96 h	-4,17	2,42	-1,72	0,1161
L	**72 h**	**-4,81**	**2,30**	**-2,09**	**0,0630**
	96 h	-4,08	2,42	-1,68	0,1230
pH	72 h	3,54	2,30	1,54	0,1542
	96 h	2,52	2,42	1,04	0,3232
S	72 h	1,74	2,30	0,76	0,4656
	96 h	0,55	2,42	0,23	0,8242
T	**72 h**	**-6,25**	**2,30**	**-2,72**	**0,0215**
	96 h	**-6,85**	**2,42**	**-2,83**	**0,0179**
A	**72 h**	**4,86**	**2,30**	**2,11**	**0,0608**
	96 h	**5,21**	**2,42**	**2,15**	**0,0569**
I/M	72 h	-1,85	2,30	0,81	0,4390
	96 h	-0,91	2,42	-0,38	0,7142

[a] EP = Erro padrão; Gli = glicose; Pep = peptona; ExM = extrato de malte; ExL = extrato de levedura; L = limoneno; S = biossurfactante; T = temperatura; A = agitação; I/M = relação inóculo/meio (m/m) [b] Parâmetros em negrito são estatisticamente significativos para a resposta (p<0,1), considerando o SS residual.

O uso de biossurfactantes como cossubstratos foi testado pela primeira vez nesse bioprocesso e, devido à falta de referências, optou-se por empregar uma concentração equivalente a uma CMC: mínima concentração a partir da qual ocorre a formação de micelas. A escolha da CMC como parâmetro de concentração foi feita em função da relação existente entre esse valor e a eficiência do surfactante (BOGNOLO, 1999; SHEPPARD, MULLIGAN, 1987). Compostos hidrofóbicos podem ser solubilizados em micelas. Tipicamente, a solubilidade do surfactante permanece baixa ate o momento em que a CMC é obtida e o aumento da concentração a valores superiores à CMC elevam sua solubilidade rapidamente e praticamente de modo linear (JÖNSSON *et al*., 1998). Assim, a principal característica do biossurfactante é a de reduzir a tensão superficial entre o meio e o substrato hidrofóbico e, assim, aumentar sua disponibilidade para o microrganismo. Para hidrocarbonetos líquidos, uma tensão superficial reduzida facilita sua emulsão, aumentando a área disponível para dissolução, a anexação microbiana e a absorção (GERSON, 1993). Paralelamente, a presença de surfactantes aumenta a permeabilidade da membrana celular com a formação de poros (CARRILLO *et al*., 2003).

Os níveis das variáveis testadas no planejamento de seleção são mostrados na Tabela 3. Esses valores codificados foram empregados na matriz Plackett-Burman PB-16 (Tabela 4). Nesse caso, um *p*-valor de 0,1 é comumente recomendado visto que ele é mais conservador e diminui o risco de falsa exclusão de parâmetros estatisticamente significativos (RODRIGUES, IEMMA, 2005).

3.1.1. Efeito da composição do meio

A composição do meio usualmente apresenta um papel importante nos processos de biotransformação, sendo um dos principais responsáveis pelas alterações no rendimento (ADAMS, DEMYTTENAERE, DE KIMPE, 2003). A presença de cossubstratos pode também melhorar a desempenho da biotransformação (TAN, DAY, 1998; SPEELMANS, BIJLSMA, EGGINK, 1998). Foi demonstrado que, considerando as amplitudes testadas, nem glicose, peptona, extrato de malte ou valor de pH apresentaram efeitos estatísticos na resposta após 72 h ou 96 h, e portanto essas variáveis não apresentam interesse significativo (p<0,1) para o processo, dentro da faixa estudada. Extrato de malte apresentou efeito negativo a 72 h (p<0,1) e a 96 h (p<0,12), o que significa que o aumento deste componente desfavorece a biotransformação de limoneno pelo fungo *F. oxysporum*, diminuindo sua atividade. Assim, de acordo com os resultados (Tabela 7), o melhor meio para este processo é a água destilada com um pH entre 5,2 e 8,2. Os experimentos seguintes foram efetuados no pH normal da água destilada. Este resultado é bastante compreensível uma vez que nos processos de biotransformação de terpenos o substrato é usualmente a única fonte de carbono e meios minerais (soluções salinas) ou tampões são tipicamente empregados como meios de cultura (MARÓSTICA JR., PASTORE, 2007b; TAN, DAY, CADWALLADER, 1998; SPEELMANS, BIJLSMA, EGGINK, 1998), apesar de que alguns autores sugerem uma relação entre melhor crescimento do fungo e melhores rendimentos de bioconversão, propondo meios com outras fontes de carbono (ADAMS, DEMYTTENAERE, DE KIMPE, 2003).

O biossurfactante de *Bacillus subtilis* não apresentou efeito estatístico (p<0,1) na resposta após 72 h ou 96 h, considerando os níveis testados. No entanto, experimentos univariados com níveis mais amplos de concentração de biossurfactante (50, 250, 1250 e 6250 $mg.L^{-1}$,

equivalentes a aproximadamente 5, 25, 125 e 625 CMCs) foram realizados em duplicata para observar o comportamento da biotransformação. Aparentemente, o rendimento aumentou até concentrações de 25 CMCs, sendo que valores mais elevados resultaram em apenas um pequeno aumento na biotransformação.

3.1.2. Efeito da concentração do substrato

Um dos maiores desafios nos processos de biotransformação consiste na elevada toxicidade e alta volatilidade do substrato e do produto (KRINGS, BERGER, 1998). Quando comparados a bactérias, os fungos são aparentemente mais sensíveis ao limoneno (BICAS; PASTORE, 2007), possivelmente devido ao fato de que esse monoterpeno aumenta a fluidez da membrana fúngica, resultando em uma permeabilidade inespecífica e perda da integridade membranar (ONKEN, BERGER, 1999b). Nesse estudo, o aumento da concentração de limoneno apresentou um efeito negativo nos níveis testados para 72 h (p<0,1) e 96 h (p<0,13) (Tabela 5), o que pode ser explicado pela ação tóxica do limoneno frente ao *F. oxysporum*. Nesse caso, menores quantidades de substrato (0,5 % ou menos) são indicadas, o que ainda permaneceria na faixa usualmente empregada nos processos de biotransformação (0,2-1,0 %) (KRINGS, BERGER, 1998). Alguns autores sugerem que a indução pelo substrato na fase de crescimento pode aumentar os rendimentos (TAN, DAY, CADWALLADER, 1998; ADAMS, DEMYTTENAERE, DE KIMPE, 2003; FONTANILLE, LARROCHE, 2003), o que não é, no entanto, o caso do presente processo (MARÓSTICA JR., PASTORE, 2007b).

O sistema testado nesse experimento, um frasco de 100 mL com tampa rosqueável de plástico, diminuiu a perda de limoneno por volatilização. Em trabalhos anteriores, o limoneno evaporou para atingir baixas concentrações em um curto período de tempo, mesmo quando

cossubstratos foram utilizados. Desconsiderando o limoneno convertido (rendimento de ~50 %), quantidades de substrato residual de menos de 10 % após 48 h (DEMYTTENAERE, VAN BELLEGHEM, DE KIMPE, 2001) e menos de 20% após 72 h foram relatadas (MARÓSTICA JR., PASTORE, 2007b). No entanto, os experimentos controle do presente trabalho apresentaram 14 %, 36 %, 57 % e 63 % de redução na área de limoneno (CG-DIC) apos 24 h, 48 h, 72 h e 96 h, respectivamente.

3.1.3. Efeito das condições de cultivo

Sabe-se que a temperatura do meio influencia diretamente reações biológicas. Ademais, a agitação do meio promove o desenvolvimento do microrganismo e as interações células-substrato. No entanto, elevadas temperaturas e agitações elevam os gastos de energia podendo favorecer reações indesejadas, perdas de substrato e produto. Assim, um balanço ideal torna-se essencial para se obter melhores resultados.

A temperatura mostrou efeito negativo (p<0,1) no processo de biotransformação (Tabela 7), explicado provavelmente pela inibição do crescimento microbiano e desnaturações enzimáticas ocorridas em temperaturas próximas a 40 °C. A Tabela 4 mostra claramente que em temperaturas de 40 °C (nível +1) praticamente não há formação de α-terpineol. Dessa forma, temperaturas mais baixas (30 °C ou menores) devem ser consideradas para se obter um melhor desempenho. Resultados similares foram obtidos com *Penicillium digitatum* NRRL 1202 (TAN; DAY; CADWALLADER, 1998) e *Pseudomonas putida* (CHATTERJEE; BHATTACHARYYA, 2001; SPEELMANS; BIJLSMA; EGGINK, 1998), que apresentaram uma redução dramática na bioconversão a temperaturas acima de 32 °C e 30 °C, respectivamente.

A agitação, por outro lado, apresentou um efeito positivo (p<0,1) na área de α-terpineol (Tabela 7), provavelmente relacionado ao aumento da interação células-substrato. Portanto, a agitação ótima pode estar situada em condições acima do máximo valor testado no planejamento de seleção de variáveis (300 rpm) e uma faixa de valores mais ampla é sugerida. Para relacionar a necessidade de agitação a fatores econômicos do processo, um nível com agitação nula (0 rpm) é exigido no DCCR.

3.1.4. Efeito do tamanho do inóculo

O tamanho do inóculo é um importante fator em uma fermentação, já que ele tem considerável efeito nos estágios subseqüentes. Em processos fermentativos industriais, é fato que a idade e densidade do inóculo influencia a duração da fase lag, a taxa de crescimento específica, o rendimento de biomassa, a esporulação, a qualidade do produto final e portanto os custos de produção (SEN; SWAMINATHAN, 2004). Uma influência positiva da quantidade de biocatalizador na quantidade de produto obtido já foi relatada para a biotransformação de α-pineno óxido em isonovalal (FONTANILLE; LARROCHE, 2003). Sugere-se também que o uso de micélios concentrados, como o empregado nesse estudo, pode aumentar significativamente o rendimento (KRINGS *et al.*, 2006). Curiosamente, o tamanho do inóculo não apresentou influência estatisticamente significativa (p<0,1) na biotransformação de *R*-(+)-limoneno a *R*-(+)-α-terpineol por *F. oxysporum*, considerando a margem testada. Assim, a mínima proporção inóculo/meio de cultivo testada (0,25, $m.m^{-1}$) foi escolhida como padrão para os ensaios posteriores.

3.2. Otimização por DCCR

De acordo com a seleção de variáveis, considerando os níveis estudados, as variáveis estudadas no DCCR foram: temperatura, agitação e concentração de substrato (limoneno). O biossurfactante aparentemente só apresenta efeitos em concentrações mais elevadas (em torno de 25 CMCs). Por esse motivo essas quatro variáveis, cujos níveis avaliados são apresentados na Tabela 5, foram otimizadas usando um DCCR acrescido de seis pontos centrais, como mostrado na Tabela 6.

Pode-se observar que os maiores aumentos na área de *R*-(+)-α-terpineol ocorreram para aqueles pontos que, apos 72 h, apresentaram respostas menores que $11 \cdot 10^6$. Todos os experimentos executados a temperaturas iguais ou inferiores a 15 °C fazem parte desse grupo, demonstrando que temperaturas mais baixas reduzem a velocidade de biotransformação e retardam o processo. Para a região de interesse (áreas maiores que $15 \cdot 10^6$), somente um aumento pequeno ou mesmo negativo na resposta foi observado após 72 h (Tabela 7). Consequentemente, de acordo com os resultados anteriores (MARÓSTICA JR.; PASTORE, 2007b), uma bioconversão de 96 h não apresentou vantagens práticas quando comparada ao processo de 72 h, o qual foi considerado ótimo tempo de biotransformação para as análises estatísticas. Esses dados (72 h) foram tratados pelo *software* STATISTICA® v. 5.5A, que gerou os coeficientes de regressão e as análises estatísticas dos parâmetros considerados (Tabela 8).

Tabela 8. Mínimos quadrados e significância dos coeficientes de regressão dos parâmetros do modelo (72 h de biotransformação) [a, b].

Parâmetros	CR ($x10^6$)	EP ($x10^6$)	t(15)	p-valor
média	**17,7**	**1,77**	**10,01**	**<0,001**
T	**6,18**	**0,89**	**6,986**	**<0,001**
T^2	**-2,48**	**0,83**	**-2,997**	**0,009**
A	**2,73**	**0,89**	**3,088**	**0,008**
A^2	**-1,97**	**0,83**	**-2,377**	**0,031**
L	-0,78	0,89	-0,886	0,390
L^2	**-3,45**	**0,83**	**-4,164**	**<0,001**
S	0,60	0,89	0,680	0,507
S^2	-0,30	0,83	-0,363	0,722
T *x* A	0,77	1,08	0,710	0,488
T *x* L	-0,89	1,08	-0,824	0,423
T *x* S	-0,81	1,08	-0,751	0,464
A *x* L	-0,04	1,08	-0,035	0,972
A *x* S	0,38	1,08	0,353	0,729
L *x* S	-0,43	1,08	-0,400	0,695

[a] CR = Coeficiente de regressão; EP = Erro padrão ; t(15) = valor t de Student para 15 graus de liberdade; T = temperatura; A = agitação; L = limoneno; S = biossurfactante. [b] parâmetros em negrito são estatisticamente significativos (p<0.05) para o modelo.

Com o objetivo de verificar a validade do modelo, uma análise de variância (ANOVA) foi feita considerando apenas as variáveis estatisticamente significativas (p<0,05) (parâmetros em negrito na Tabela 8).

Tabela 9. ANOVA do modelo quadrático [a].

Fonte de variação	SQ ($x10^{14}$)	gl	QM ($x10^{13}$)	F calculado	p-valor
Regressão	16,9	5	33,4	23,5	< 0.0001
Resíduos	3,46	24	1,44		
Total	20,4	29			
R^2 = 0,83				$F_{0.95(5,24)}$ = 2,62	

[a] SQ = Soma dos quadrados; gl = graus de liberdade; QM: Quadrados médios.

A tabela de ANOVA (Tabela 9) demonstrou que o modelo quadrático ajustado para as respostas do processo é satisfatório. O valor de F calculado mostrou-se nove vezes maior que o respectivo valor tabelado, enquanto que o p-valor do modelo mostrou-se menor que 0,0001. Apesar de não ideal, o valor de R^2=0,83 é perfeitamente aceitável para sistemas biológicos (RODRIGUES; IEMMA, 2005). Consequentemente, foi possível definir o modelo codificado dado pela Equação 1, que relaciona a resposta avaliada em função das variáveis estudadas:

$$\text{Área } \alpha T\ (x10^6) = 17,37 + 6,18 \cdot T - 2,44 \cdot T^2 + 2,73 \cdot A - 1,93 \cdot A^2 - 3,40 \cdot L^2 \quad \text{(Eq. 1)}$$

Onde

Área αT, T, A e L são, respectivamente, a área do pico de *R*-(+)-α-terpineol em GC-DIC, temperatura, agitação e concentração de limoneno em valores codificados (Tabela 6).

Essa equação pode ser graficamente representada pelas curvas de contorno Agitação x Temperatura (Figura 10), Limoneno x Temperatura (Figura 11) e Limoneno x Agitação (Figura 12). O perfil das superfícies de resposta obtidas é ideal, visto que todas as figuras apresentam as regiões ótimas preditas dentro dos níveis estudados. Uma análise da Equação 1 mostra que quando a concentração de limoneno é fixada em 0,5 % (v/m) a temperatura e a agitação podem variar de 23 a 29,5 °C e de 180 a 310 rpm, respectivamente, mantendo a produção acima de 95% do valor máximo. Em um processo não agitado a máxima concentração de *R*-(+)-α-terpineol pode atingir 1 $g.L^{-1}$. As condições ótimas foram obtidas através de métodos matemáticos (derivação da equação). Nesse caso os valores obtidos foram: temperatura ≈ 26 °C, agitação ≈ 240 rpm e concentração de limoneno = 0,5% (v/m), com uma área predita de *R*-(+)-α-terpineol de $22,25 \cdot 10^6$, equivalente a 2,44 g de *R*-(+)-α-terpineol por litro de meio [$\alpha T_{g.L^{-1}}$ = (Área αT + $1,57 \cdot 10^6$)/$9,76 \cdot 10^6$; R^2 = 0,992]. Devido a razões práticas, optou-se por utilizar 30 °C, 200 rpm e 0,5 % de substrato como padrão, mantendo a produção próxima à máxima.

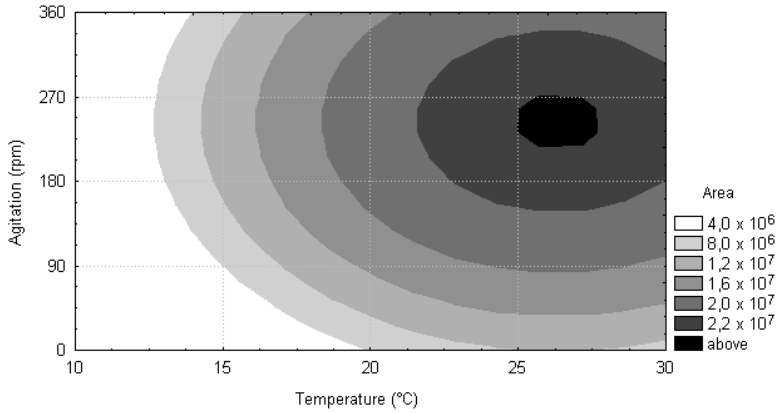


Figura 10. Superfície de contorno da área de *R*-(+)-α-terpineol após 72 h de biotransformação em função da agitação e temperatura. Concentração de limoneno fixada em 0,5% (v/m).

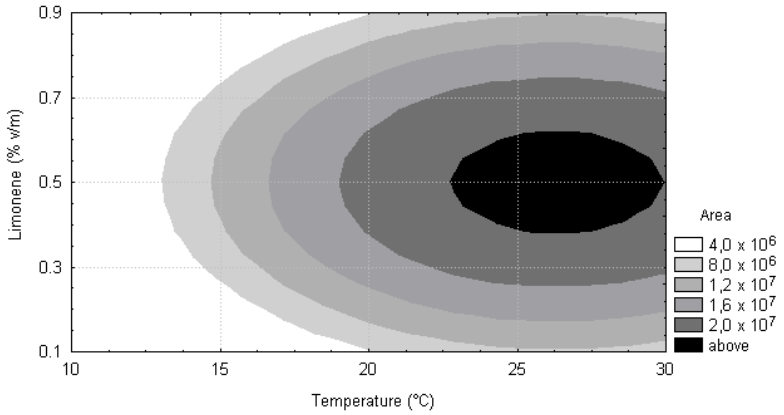


Figura 11. Superfície de contorno da área de *R*-(+)-α-terpineol Após 72 h de biotransformação em função da concentração de limoneno e temperatura. Agitação fixada em 180 rpm.

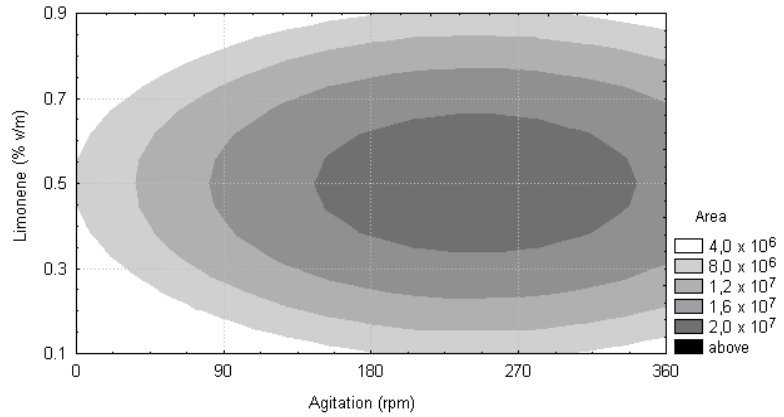


Figura 12. Superfície de contorno da área de *R*-(+)-α-terpineol após 72 h de biotransformação em função da concentração de limoneno e agitação. Temperatura fixada em 20°C.

4. Conclusão

Pela primeira vez uma seleção de variáveis seguida por uma otimização por DCCR foi descrita para a otimização dos principais processos envolvidos na biotransformação do *R*-(+)-limoneno a *R*-(+)-α-terpineol. O efeito da presença de um biossurfactante nesse processo foi um relato adicional. Essa técnica de otimização mostrou-se bastante útil para uma boa compreensão do processo. De acordo com as superfícies de resposta as melhores condições para a maior recuperação de *R*-(+)-α-terpineol foram: 72 h de reação em um meio contendo três partes de água destilada pura e uma parte de inóculo (concentrado micelar), inoculado a uma temperatura entre 24 e 28 °C, agitação de 200 a 310 rpm e concentração de substrato (*R*-(+)-limoneno) de 0,5 % (v/m). Esse é um processo simples e barato com uma produção relativamente elevada de *R*-(+)-α-terpineol (2,4 $g.L^{-1}$) que representa o primeiro passo para o desenvolvimento de um processo industrial. As etapas subseqüentes dessa pesquisa devem ser estudos com o inóculo (imobilização, liofilização, permeabilização da membrana), uso de meios bifásicos além de estudos de *scale-up* e recuperação/separação do produto. No futuro, uma possível manipulação genética do microrganismo deve ser considerada.

CAPÍTULO 4

COPRODUÇÃO DE LIPASE ALCALINA E *R*-(+)-α-TERPINEOL PELO FUNGO *FUSARIUM OXYSPORUM*

1. Introdução

Lipases (E.C. 3.1.1.3) são enzimas que catalisam a hidrólise de triglicerídeos para di e monoglicerídeos, glicerol e ácidos graxos. Em condições de baixa atividade de água, essas enzimas catalisam as reações inversas (SHARMA; CHISTI; BANERJEE, 2001). Suas principais aplicações são no processamento de alimentos (RAJMOHAN; DODD; WAITES, 2002), produção de detergentes (SAISUBRAMANIAN *et al*., 2006), processamento de óleos (YASSIN *et al*., 2003), biocatálise de compostos enantiomericamente puros (GOTOR-FERNÁNDEZ; BRIEVA; GOTOR, 2006) além de síntese de compostos de aroma (MACEDO; LOZANO; PASTORE, 2003, MELO; PASTORE; MACEDO, 2005). Lipases alcalinas formam um grupo que reúne as enzimas com atividade ótima em valores de pH elevados. Esse grupo tem recebido atenção especial nos últimos anos devido a seu amplo potencial de aplicação, especialmente para indústria de aromas. Diversos microrganismos têm sido descritos como produtores desse grupo de lipases, entre eles *Pseudomonas* sp. (LIN; CHIOU; TSAI, 1995), *Acinetobacter radioresistens* (CHEN; CHENG; CHEN, 1998), *Bacillus* sp. (ERTUĞRUL; DÖNMEZ; TAKAÇ, 2007, SHARMA *et al*., 2002), *Penicillium* sp. (LIMA *et al*., 2004) e *Aspergillus carneus* (SAXENA *et al*., 2003).

Muitos trabalhos relatam a produção de enzimas por espécies do gênero *Fusarium*, como *F. graminearum* (VOIGT; SCHAEFER; SALOMON, 2005), *F. solani* (MAIA *et al*., 2001) e *F.*

oxysporum (PIO; MACEDO, 2007). Recentemente, a linhagem *F. oxysporum* 152b foi selecionada devido a sua elevada produção de lipase alcalina extracelular (PRAZERES, 2006). Ao mesmo tempo, outras publicações também descreveram a biotransformação de *R*-(+)-limoneno para *R*-(+)-α-terpineol pela mesma linhagem (BICAS *et al*. 2008a; MARÓSTICA JR.; PASTORE, 2007b). No entanto, as principais características da enzima responsável pelo processo ainda não foram descritas. Nesse capítulo, a biotransformação do *R*-(+)-limoneno para *R*-(+)-α-terpineol foi parcialmente caracterizada e um processo integrado para a coprodução de lipase/*R*-(+)-α-terpineol foi posteriormente proposto.

2. Material e Métodos

2.1. Microrganismos e reagentes

Nesse estudo os padrões de terpenos utilizados foram: *R*-(+)-limoneno (Fluka, ≥96% pureza), S-(–)-limoneno (Aldrich, 96% pureza), α-terpineol (Aldrich, 90% pureza), *R*-(+)-α-terpineol (Fluka, ~99% pureza) e *p*-nitrofenil laurato (*p*-NPL) (ICN Biomedicals Inc.). O microrganismo *Fusarium oxysporum* 152b foi isolado de frutas do nordeste (PRAZERES, 2006) e mantido no banco de cepas do Laboratório de Bioaromas – FEA/UNICAMP.

2.2. Preparo do inóculo (biomassa)

Uma peça de ágar (~1,5 cm^2) contendo a cultura de *F. oxysporum* 152b com idade de 48 h foi transferida para um frasco cônico de 250 mL contendo 50 mL de meio YM (em $g.L^{-1}$: glicose = 10, peptona = 5, extrato de levedura = 3, extrato de malte = 3). O material foi homogeneizado em condições estéreis com um Ultra-Turrax® T18 até completa ruptura da fração sólida. Após inoculação por 72 h a 30°C e 160 rpm a massa celular foi recuperada por filtração a vácuo em um

funil de Buchner com papel de filtro Whatman n° 1 (BICAS *et al*., 2008a). Essa biomassa foi empregada diretamente (biomassa fresca), congelada ou liofilizada antes de ser empregada nos ensaios de biotransformação.

2.3. Procedimento de biotransformação

A biomassa obtida no item anterior (3,75 g para biomassa fresca ou congelada e 50 mg para biomassa liofilizada) foi distribuída em frascos estéreis de 100 mL com tampa rosqueável. Em seguida, água destilada e 75 µL de substrato (0,5 %, $v.m^{-1}$) foram adicionados para totalizar uma massa final de 15 g. Os frascos foram incubados a 30°C / 200 rpm e amostras de 1 mL foram retiradas periodicamente (BICAS *et al*., 2008a).

Para a biotransformação anaeróbica 62,5 g de biomassa fresca (inóculo), 187,5 g de água destilada e 1.250 µL de substrato (0,5 %, $v.m^{-1}$) foram transferidos para um frasco cônico de 500 mL com duas entradas (uma na base e outra no gargalo). Durante 5 min, antes do início do processo ou após a retirada de cada amostra, nitrogênio gasoso foi borbulhado pela base para retirar todo oxigênio dissolvido no meio e aquele do *headspace*. O frasco foi incubado a 30°C / 200 rpm e amostras de 1 mL foram retiradas periodicamente.

2.4. Determinação e quantificação dos compostos de aroma

Cada amostra foi extraída (40 s em *Vortex*) com o mesmo volume de acetato de etila. Após a separação de fases, a fração orgânica foi diretamente injetada (1 µL) em modo split (razão de split = 1:10) em um cromatógrafo gasoso com detector por ionização de chama (CG-FID) HP 7890 equipado com uma coluna HP-5 (Agilent) de 30 m x 0,32 mm i.d. x 0,25 µm espessura de filme. A temperatura do forno foi mantida a 80°C por 3 min, elevada a $20°C.min^{-1}$ até 200°C, a qual foi mantida por mais 4 min. As temperaturas do injetor e do detector foram mantidas a

250°C. O limoneno e o α-terpineol foram quantificados por uma curva de calibração contendo concentrações de ambos padrões, utilizando *n*-decano como padrão interno. Todos os experimentos foram efetuados em triplicata (adaptado de BICAS *et al*., 2008b).

2.5. Procedimento de produção de lipase

Uma peça de ágar (~1,5 cm^2) contendo a cultura de *F. oxysporum* 152b com idade de 48 h foi transferida para um frasco cônico de 250 mL contendo 50mL de meio sintético (em $g.L^{-1}$: peptona = 15, óleo de oliva = 10, extrato de malte = 5; K_2HPO_4 = 3, $MgSO_4.7H_2O$ = 0,4; pH 6,0). O material foi homogeneizado em condições estéreis com um Ultra-Turrax® T18 até completa ruptura da fração sólida. O então chamado inóculo foi resultado da incubação desse microrganismo por 24 h a 30°C e 160 rpm. Uma alíquota de 1.000 µL desse inóculo (~1.10^6 $UFC.mL^{-1}$) foi transferida para um frasco cônico de 250 mL contendo 50 mL do mesmo meio sintético descrito acima, sendo o frasco posteriormente mantido a 30°C / 160 rpm. Amostras de 1.000 µL foram assepticamente retiradas periodicamente (0, 24, 48, 72, 100 h), centrifugadas a 20.000 *g* por 15 minutos e o extrato enzimático bruto (sobrenadante) foi utilizado para a avaliação da atividade enzimática.

Para a determinação da atividade enzimática empregou-se como substrato 161 mg de *p*-NPL dissolvidos em 80 mL de tampão acetato 50 mM em pH 5,6 com 1 % de triton X-100, mantido a 55°C. Para a reação adicionou-se 500 mL de substrato, 450 µL de tampão Tris-HCl 50 mM em pH 8,0 e 50 µL de amostra (extrato enzimático bruto). Após 10 min a 55 °C, a reação foi paralisada pela adição de 2 mL de etanol anidro. A absorbância a 410 ηm foi avaliada 10 minutos depois. Uma unidade de atividade enzimática ($U.mL^{-1}$) foi definida como µmols de *p*-nitrofenol produzido por minuto por mL de extrato enzimático bruto adicionado. Os resultados se basearam

em uma curva padrão de *p*-nitrofenol feita sob as mesmas condições. Todos os ensaios foram feitos em triplicata (PRAZERES, 2006).

3. Resultados e Discussão

3.1. Biotransformação convencional

A biotransformação convencional foi efetuada conforme o método descrito por Maróstica Jr. e Pastore (2007b) e otimizado por Bicas *et al*. (2008a). Nas condições propostas, a produção atingiria cerca de 2,4 $g.L^{-1}$ após 72 h de cultivo. Ao se repetir este procedimento, obteve-se a curva de produção (Figura 13) que mostra uma produção de α-terpineol de quase 4 $g.L^{-1}$ em 48 h, que permanece estável até 96h de cultivo. Essa maior produção pode ser explicada pelo método de extração empregado anteriormente (microextração em fase sólida ou SPME), cuja eficiência de extração não fora determinada. No presente estudo, a eficiência de extração para o limoneno e o α-terpineol em acetato de etila foram de 16,5 % e de 32,6 %, respectivamente (dados não apresentados). Assim, os valores (reais) corrigidos mostraram-se substancialmente maiores.

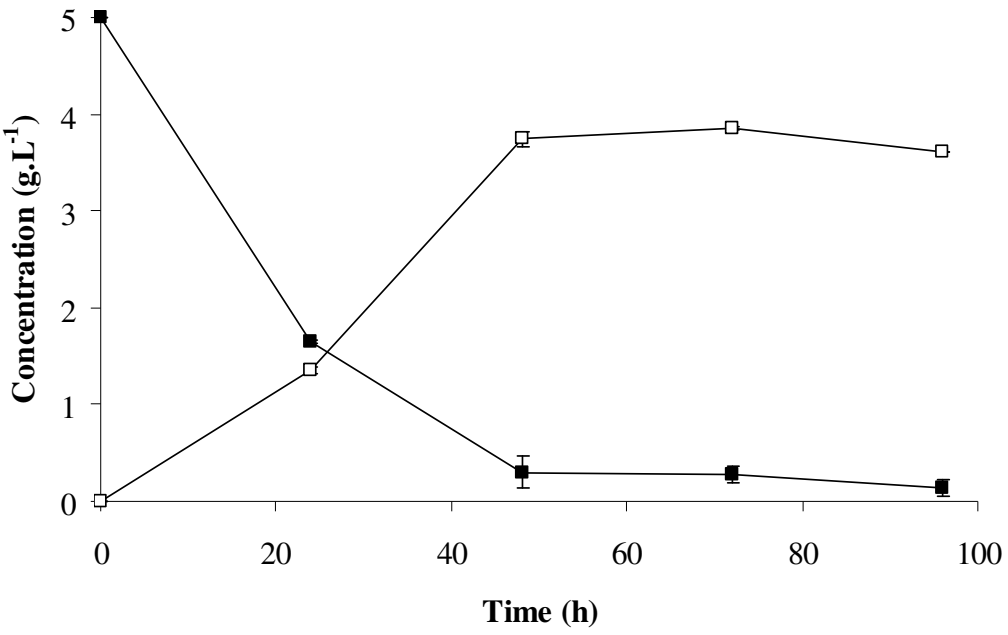


Figura 13. Biotransformação de *R*-(+)-limoneno (■) para *R*-(+)-α-terpineol (□)em *shaker* (30 °C / 200 rpm) pela biomassa fresca do fungo *F. oxysporum* 152b desenvolvida em meio YM.

3.2. Biotransformação com biomassa congelada ou liofilizada

O processo de congelamento com posterior descongelamento de um microrganismo resulta em uma semi-permeabilização da biomassa, liberando parcialmente enzimas intracelulares (FONTANILLE; LARROCHE, 2003). A Figura 14 mostra que a biotransformação do *R*-(+)-limoneno para *R*-(+)-α-terpineol ocorre com biomassa congelada de *F. oxysporum* 152b. Ainda, quando comparado ao processo convencional (Figura 13), essa reação foi acelerada, o que realmente indica a liberação da enzima responsável pela conversão e, dessa forma, comprova sua natureza intracelular. De fato, o filtrado resultante da separação da massa celular (vide Material e métodos), onde estariam presentes enzimas extracelulares, não apresentou nenhuma atividade para produção de α-terpineol (dados não apresentados). Por outro lado, o microrganismo na

forma liofilizada também apresentou atividade biotransformadora, o que pode ser interessante do ponto de vista técnico por padronizar, simplificar a inoculação e possibilitar o estoque do biocatalisador. No entanto, a concentração máxima de produto mostrou-se cerca de 40 % inferior (Figura 14). Esse menor desempenho não pode ser explicado pela baixa concentração de liofilizado, visto que o uso de quantidades 10 vezes superiores (0,5 g), não foi suficiente para elevar a produção (resultados não apresentados). Assim, imagina-se que o processo de liofilização danifica de alguma forma a enzima reduzindo sua atividade de conversão do limoneno.

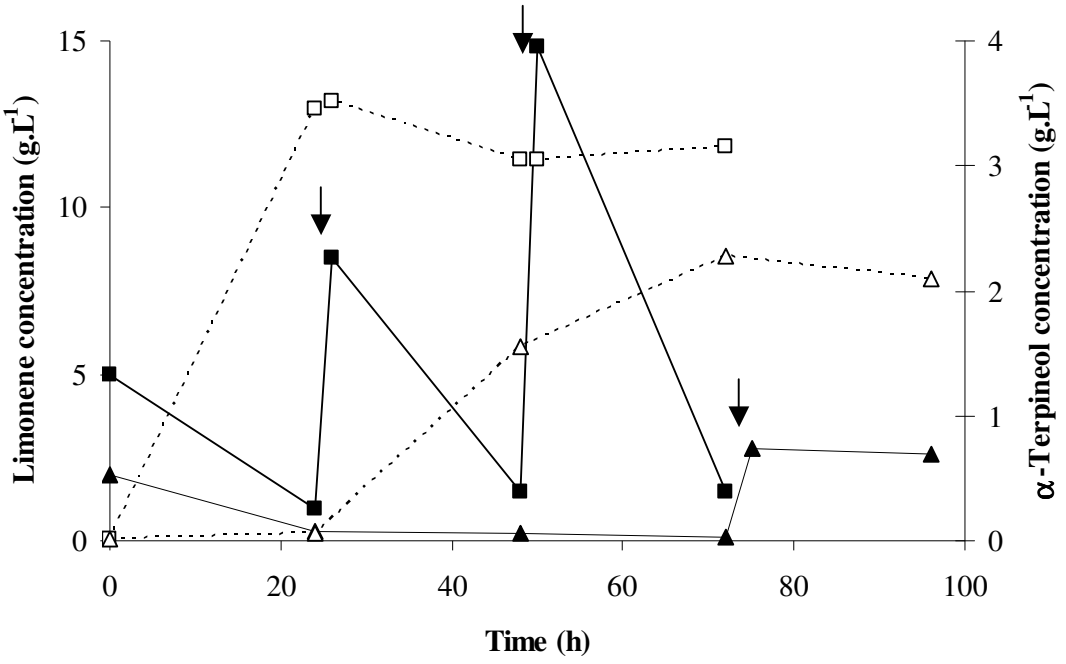


Figura 14. Biotransformação de *R*-(+)-limoneno (símbolos negros) para *R*-(+)-α-terpineol (símbolos brancos) em *shaker* (30 °C / 200 rpm) pela biomassa congelada (quadrados) ou liofilizada (triângulos) do fungo *F. oxysporum* 152b desenvolvidas em meio YM. As setas representam o momento de adição de substrato.

3.3. Caracterização da biotransformação de *R*-(+)-limoneno para *R*-(+)-α-terpineol

A biotransformação em sistema anaeróbico é uma das ferramentas para caracterizar a enzima envolvida nos processos em questão, especialmente no que se refere à exigência de cofatores (BICAS *et al*., 2008b). Do ponto de vista técnico, essa informação também é muito valiosa por determinar a necessidade de aeração do sistema. A Figura 15 indica que a biotransformação de *R*-(+)-limoneno para *R*-(+)-α-terpineol, embora ocorrida em menor taxa, é catalisada por uma enzima independente de oxigênio e consequentemente de cofatores. A independência de cofatores também estaria relacionada ao aumento de velocidade no procedimento com biomassa congelada/descongelada (Figura 14), pois a enzima intracelular liberada para o meio, fato responsável pelo aumento de velocidade, só apresentaria ação se independesse da presença de cofatores.

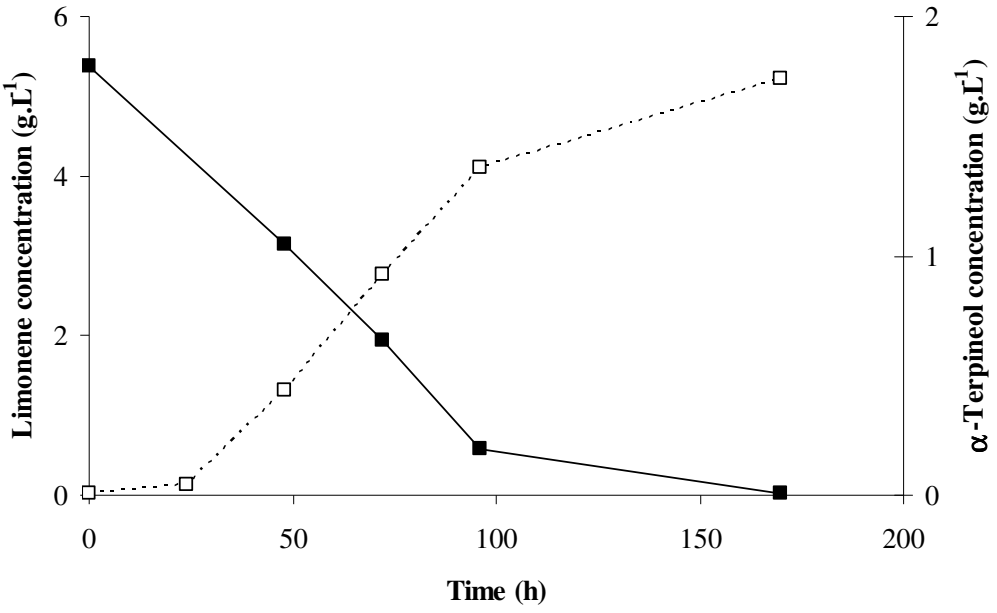


Figura 15. Biotransformação de *R*-(+)-limoneno (■) para *R*-(+)-α-terpineol (□) em *shaker* (30 °C / 200 rpm) e condições anaeróbicas (vide Materal e métodos) pela biomassa fresca do fungo *F. oxysporum* 152b desenvolvida em meio YM.

Ensaios com os isômeros *R*-(+)- e *S*-(–)-limoneno demonstram que ambos isômeros são convertidos para, respectivamente, *R*-(+)- e *S*-(–)-α-terpineol. Todavia, a atividade obtida com o isômero *R*-(+) chega a ser mais de 10 vezes superior (Figura 16), algo muito semelhante ao observado por Braddock e Cadwallader (1995) com a bactéria *Pseudomonas gladioli*. Portanto, a enzima responsável pela biotransformação do limoneno mostrou-se enantioespecífica e razoavelmente enantioseletiva para a conversão de *R*-(+)-limoneno para *R*-(+)-α-terpineol. Contrariamente, a bactéria *Sphingobium* sp. (*P. fluorescens* NCIMB 11671) mostrou-se menos seletiva, pois converteu ambos *R*-(+)- e *S*-(–)-enantiômeros para, respectivamente, *R*-(+)- e *S*-(–)-α-terpineol com praticamente a mesma eficiência, como será abordado em mais detalhes no Capítulo 6 dessa tese.

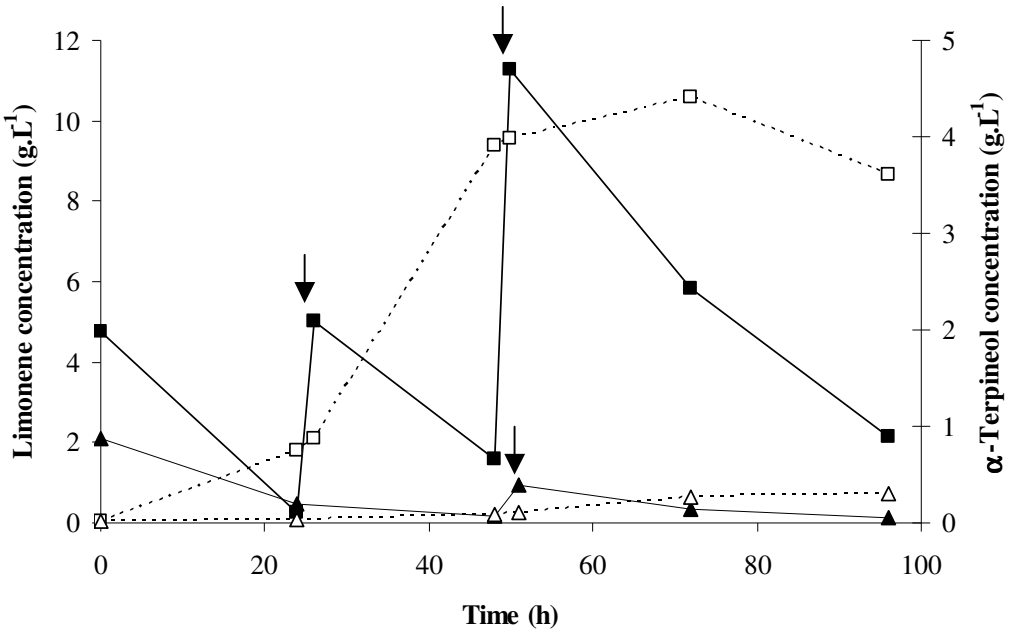


Figura 16. Biotransformação de *R*-(+)-limoneno (■) para *R*-(+)-α-terpineol (□) e de *S*-(–)-limoneno (▲) para *S*-(–)-α-terpineol (△) em *shaker* (30 °C / 200 rpm) pela biomassa fresca do fungo *F. oxysporum* 152b desenvolvida em meio YM. As setas representam o momento de adição de substrato.

Ao que tudo indica, a produção de *R*-(+)-α-terpineol apresentou um limite máximo, ao redor de 4,0 $g.L^{-1}$, devido à inibição pelo próprio produto. A adição de mais substrato não resultou em retomada na produção (Figuras 14 e 16), o que sugere não ter havido limitação por baixa concentração de substrato, como ocorrido para *Sphingobium* sp (vide Capitulo 6). Por outro lado, mesmo em processos mais rápidos, como no caso de biomassa congelada/descongelada (Figura 13), o limite máximo é o mesmo, demonstrando não se tratar de uma perda de atividade ao longo do tempo. Dessa forma, a única maneira de aumentar ainda mais a produção do álcool terpênico seria a completa renovação do meio de cultura, o que muitas vezes se torna desinteressante do ponto de vista industrial, ou o emprego de sistemas bifásicos. No entanto, a linhagem em questão não foi capaz de proceder a transformação de limoneno em sistemas bifásicos, independentemente da condição da biomassa (fresca ou congelada), das condições da reação (quantidade de substrato, agitação) ou da fase orgânica empregada (*n*-hexadecano, óleo de soja) (resultados não apresentados).

3.4. Processo integrado de produção de lipase e *R*-(+)-α-terpineol

A linhagem *F. oxysporum* 152b já foi descrita como produtora de lipase alcalina extracelular (PRAZERES, 2006). Estudos ainda não publicados também demonstraram que sua produção ocorria em presença de fontes alternativas de óleos (agentes indutores) e meios de cultivo. Conforme apresentado na Figura 17, a produção máxima de lipase (cerca de 14 $U.mL^{-1}$) ocorreu em torno de 72 h de cultivo. Ao mesmo tempo, outros trabalhos relataram sua capacidade de biotransformar o *R*-(+)-limoneno para *R*-(+)-α-terpineol partindo-se de um inóculo com 72 h de idade (MARÓSTICA JR.; PASTORE, 2007b; BICAS *et al*., 2008a). Porém, como esses processos lidam com enzimas de natureza distinta (lipase extracelular e enzima intracelular para

biotransformação do limoneno), a biomassa resultante do processo de produção de lipase era descartada ao passo que para a produção de α-terpineol somente ela interessava. Portanto, para a integração dos processos restava demonstrar se o microrganismo cultivado em meio sintético de produção de lipase mantinha-se ativo para a biotransformação do limoneno.

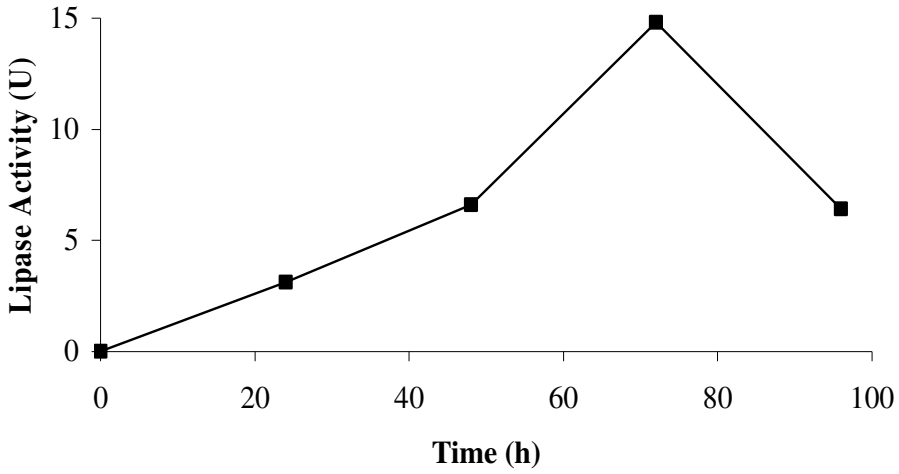


Figura 17. *Time-course* de produção de lipase alcalina extracelular de *F. oxysporum* 152b cultivado em meio sintético a 30 °C e 160 rpm.

No processo de coprodução proposto a atividade de lipase obtida foi de 14,2 ± 0,8 $U.mL^{-1}$ (72 h), equivalente ao apresentado na Figura 17. Também foi comprovado, como observado na Figura 18, que a biomassa resultante desse processo manteve a capacidade de biotransformar o limoneno, apesar de que a máxima concentração de α-terpineol obtida foi de cerca de 50 % inferior quando comparada à do procedimento convencional (Figura 13). Dessa forma, sugere-se que a fonte lipídica empregada no meio sintético de produção de lipase pode de alguma forma prejudicar a degradação do limoneno, talvez por um mecanismo semelhante à repressão

catabolítica que seria conferida pelos resíduos de óleo na biomassa. Outra hipótese seria a anaerobiose conferida pelos resíduos de óleo, que fariam uma micro barreira ao redor do biocatalisador impedindo o acesso ao oxigênio atmosférico (para maiores detalhes, vide Capítulo 6). Isso explicaria as semelhanças entre as Figuras 15 e 18.

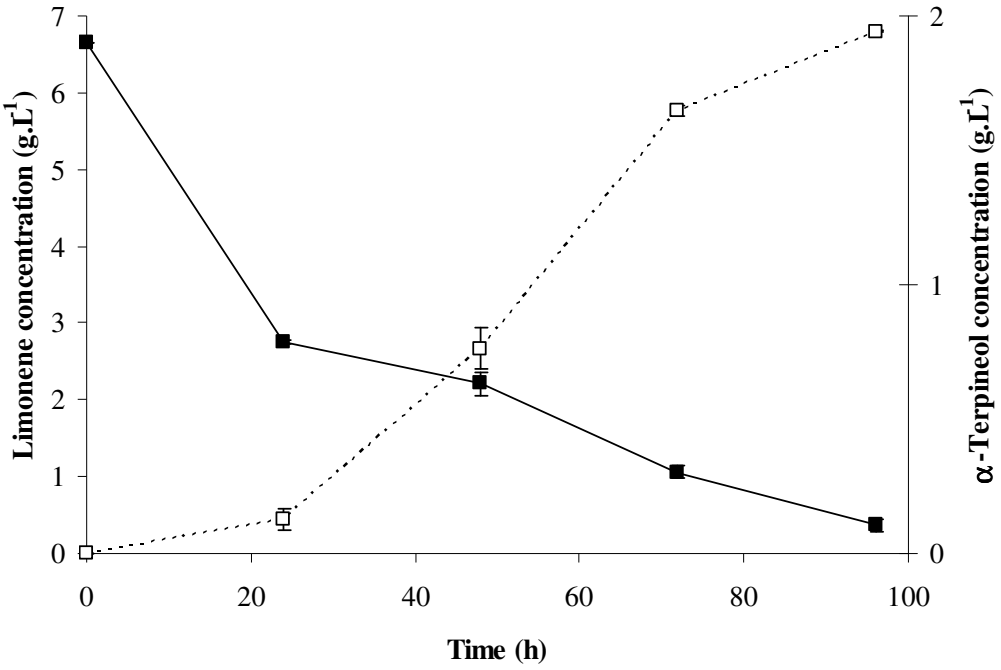


Figura 18. Biotransformação de *R*-(+)-limoneno (■) para *R*-(+)-α-terpineol (□) em *shaker* (30 °C / 200 rpm) pela biomassa fresca do fungo *F. oxysporum* 152b desenvolvida em meio sintético para produção de lipase.

4. Conclusão

Esse trabalho foi um complemento a uma série de estudos com a linhagem fúngica *Fusarium oxysporum* 152b, no qual foram descritas alguma das características da enzima e do processo de biotransformação do limoneno para o composto de aroma *R*-(+)-α-terpineol. Foi demonstrado que a enzima responsável por tal conversão é estereoespecífica e seletiva para a biotransformação de *R*-(+)-limoneno para *R*-(+)-α-terpineol, é de natureza intracelular e capaz de atuar em ausência de oxigênio. A biomassa empregada nesse processo pode ainda ser estocada congelada ou liofilizada, sendo que no primeiro caso a conversão foi significativamente acelerada. Por fim, demonstrou-se ser possível empregar a biomassa resultante do processo de produção de lipase para biotransformar o *R*-(+)-limoneno. Assim, apesar de os resultados obtidos ainda serem insuficientes para um aproveitamento industrial, esse estudo abre perspectivas a futuros trabalhos de produção integrada para a completa exploração do potencial biotecnológico dos microrganismos.

CAPÍTULO 5

CHARACTERIZATION OF THE METABOLIC PATHWAYS FOR SELECTED MONOTERPENES IN TWO SPECIES OF *PSEUDOMONAS*

Reproduced in part from Bicas, J. L.; Fontanille, P.; Pastore, G. M.; Larroche, C. (2008) Journal of Applied Microbiology. 105:1991-2001.

1. Introduction

The reaction of α-pinene degradation described in the first studies passed through limonene and perillyl alcohol to form isopropyl pimelic acid, besides four other possible pathways (SHUKLA; BHATTACHARYYA, 1968; SHUKLA; MOHOLAY; BHATTACHARYYA, 1968). Some years later, other authors described another α-pinene degradation pathway passing by limonene but with other intermediate acids (GIBBON; PIRT 1971; TUDROSZEN; KELLY; MILLIS, 1977). A third metabolic route (pathway 1a in Figure 19), a variation of the last, was proposed for *Pseudomonas fluorescens* NCIMB 11671, in which α-pinene was completely degraded (BEST *et al.*, 1987). A similar pathway was also verified for *Nocardia* sp. (GRIFFITHS *et al.*, 1987a,b). Further reports suggest a different dynamic for this pathway, explaining the formation of novalal by isomerization of isonovalal (LAROCHE; FONTANILLE; LARROCHE, 2006; ZORN; NEUSER; BERGER, 2004) (pathway 1b in Figure 19). Studying this process more in depth, an optimized method for isonovalal production from α-pinene oxide by *P. rhodesiae* has been developed (FONTANILLE; LE FLÈCHE; LARROCHE, 2002; FONTANILLE; LARROCHE 2003). More recently, Yoo and Day (2002) reported another α-pinene degradation pathway for *Pseudomonas* PIN. This novel route integrates α-pinene, β-pinene, limonene and *p*-cymene and leads to the formation of perillic acid, cumic acid and α-terpineol.

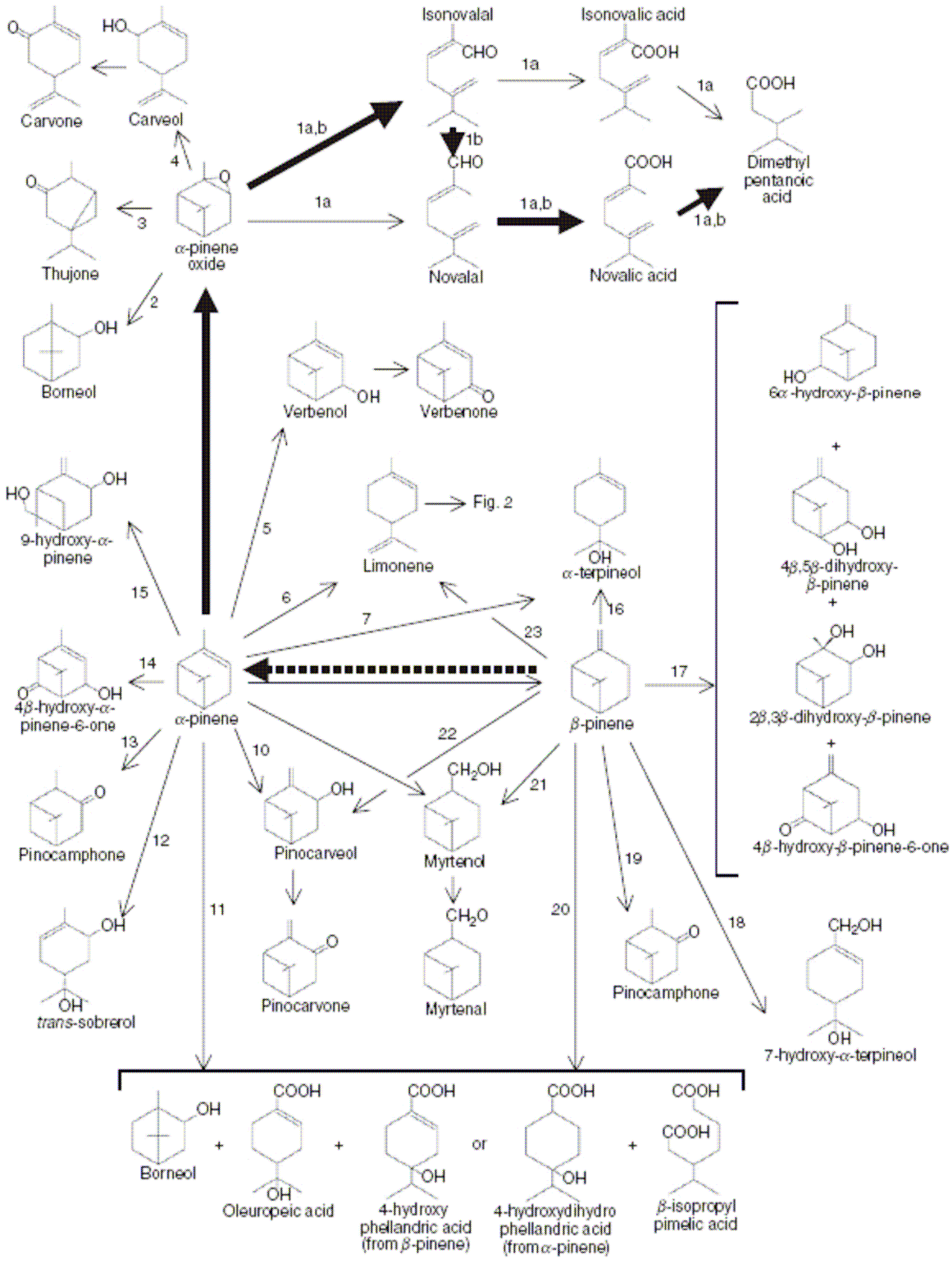


Figure 19. Main metabolic pathways of α- and β-pinene described in the literature. Pathways: **1a** reported by Best *et al.* (1987) and Griffiths *et al.* (1987a,b); **1b** by Laroche, Fontanille and

Larroche (2006); Zorn, Neuser and Berger (2004); **2-4** and **13-15** by Schrader (2007); **5** by Agrawal, Deepika and Joseph (1999); Agrawal and Joseph (2000a,b); Draczynska (1985); Prema and Bhattacharyya (1962a); Rozenbaum *et al*. (2006); Van Dyk, Van Rensburg and Moleleki (1998); and Wright *et al*. (1986); **6** by Gibbon and Pirt (1971); Savithiry *et al*. (1998); Tudroszen, Kelly and Millis (1977) and Yoo and Day (2002); **7** by Chatterjee, De and Bhattacharyya (1999); Draczynska *et al.* (1985); Rozenbaum *et al.* (2006) and Wright *et al*. (1986); **8-10**, **21** and **22** by Savithiry *et al*. (1998); **11** and **20** by Shukla and Bhattacharyya (1968) and Shukla, Moholay and Bhattacharyya (1968); **12** by Draczynska *et al*. (1985); Prema and Bhattacharyya (1962a) and Wright *et al*. (1986); **16** by Rozenbaum *et al*. (2006) and Toniazzo *et al*. (2005); **17** by Farooq *et al*. (2002); **18** by Draczynska *et al.* (1985); **19** by Van Dyk, Van Rensburg and Moleleki (1998) and **23** by Savithiry *et al*. (1998) and Yoo and Day (2002). The wide arrows represent the metabolism of pinenes by *P. rhodesiae* and *P. fluorescens* and the dash arrows correspond to the isomerization described in this study.

The degradation pathways for β-pinene have not yet been fully elucidated. It is known that some *Aspergillus niger* strains are capable of transforming it into α-terpineol as main product (TONIAZZO *et al.*, 2005; ROZENBAUM *et al.*, 2006). Different hydroxylated products have also been identified for *Botrytis cinerea* (FAROOQ *et al.*, 2002) and *Armillariella mellea* (DRACZYŃSKA *et al.*, 1985) (pathways 17 and 18 in Figure 19). For bacteria, a common route for limonene, α- and β-pinene has already been described for *Bacillus pallidus* BR425. In this case, myrtenol and pinocarveol are the main products from β-pinene (SAVITHIRY *et al.* 1998). Conversely, the fermentation of β-pinene by *Pseudomonas* PL, resulted in different products (SHUKLA; BHATTACHARYYA, 1968) (pathway 20 in Figure 19). The main metabolic pathways for α- and β-pinene are summarized in Figure 19.

It is observed that limonene is commonly found as an intermediate in the metabolism of α- and β-pinene. Therefore, it is possible to assume that, for some microorganisms, these two substrates may also follow a pathway similar to that observed for limonene. Maróstica and Pastore (2007a) have recently reviewed the main metabolic routes for limonene and Figure 20 illustrates its six different degradation pathways, which are 1) oxidation of carbon 7 to perillyl compounds; 2) ring double bond epoxidation, followed by the corresponding diol formation and its oxidation; 3) carbon 6 oxidation to form carveol, carvone and dihydrocarvone; 4) carbon 8 hydroxylation to directly form α-terpineol; 5) oxidation of carbon 3 to form isopiperitenol and isopiperitenone and 6) 8,9 double bond epoxidation to form limonene-8,9-epoxide. Other papers describe the catabolism of acyclic monoterpenes (FÖRSTER-FROMME *et al*., 2006 and references therein), the degradation of monoterpenes in anaerobic conditions (HARDER; PROBIAN 1995) and review the biotransformation of limonene (DUETZ *et al*., 2003) and other terpenes (VAN DER WERF; DE BONT J; LEAK, 1997; DE CARVALHO; DA FONSECA 2006a).

The two strains tested in this study, *Pseudomonas rhodesiae* CIP 107491 and *P. fluorescens* NCIMB 11671, are recognized for their ability to use α-pinene as sole carbon source and to convert it into different oxygenated terpenoids. However, the information on the utilization of other terpenes is limited. Therefore, different terpene sources were tested as sole carbon sources and as substrates for biotransformation for these microorganisms.

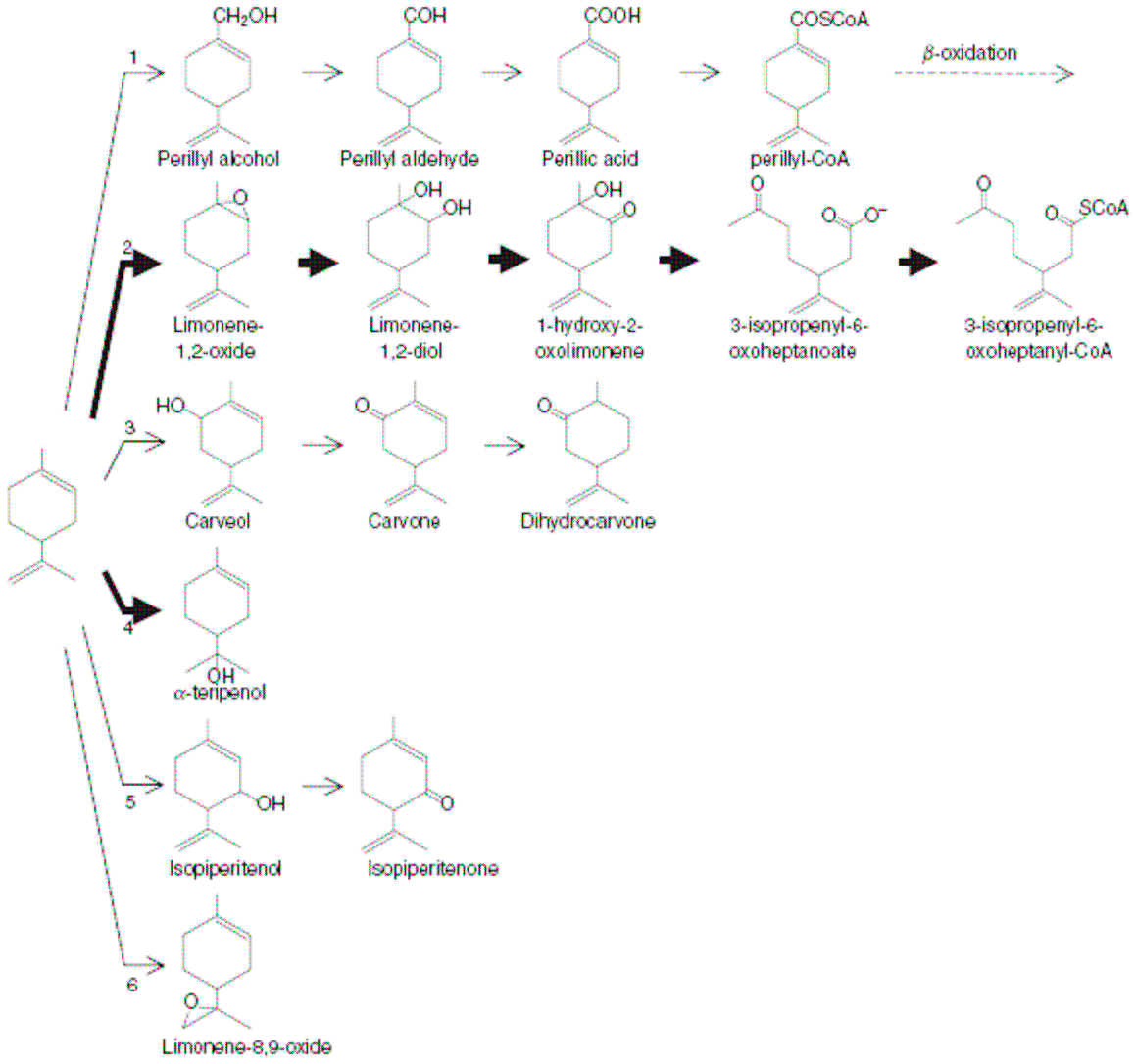


Figure 20. The six main metabolic pathways for limonene (MARÓSTICA; PASTORE 2007a; VAN DER WERF; SWARTS; DE BONT, 1999). The wide arrows represent the metabolism of limonene by *P. fluorescens* described in this study.

2. Materials and methods

2.1. Microorganisms and chemicals

The two strains employed in this work were *Pseudomonas rhodesiae* CIP107491 and *P. fluorescens* NCIMB 11671. Alpha-pinene (Acros, 97% purity), (–)-β-pinene (Fluka, >99% purity), R-(+)-limonene (Fluka, ~98% purity), (–)-α-pinene oxide (Aldrich, 97% purity), β-pinene oxide (Acros, mixture of *cis/trans* isomers, 75% purity), (+)-limonene-1,2-oxide (Aldrich, mixture of cis/trans isomers, 97% purity) and (+)-carvone (Acros, 98% purity) were used as substrates in *n*-hexadecane (SDS, 99% purity) as organic phase. The complex monoterpene mixtures turpentine and orange peel oil, obtained in the Brazilian market, were also tested.

2.2. Pre-culture preparation

Three full loops of a 24 h-old culture on a Petri dish were transferred to a 500 mL conical flask which contained 1.0 g carbon source (sodium lactate for *P. rhodesiae* and glucose for *P. fluorescens*), 0.25 g $(NH)_4SO_4$, 5 mL Hutner solution (Table 10), 10 mL solution A (6.5 g K_2HPO_4, 8.28 g KH_2PO_4 in 250 mL distilled water) and 235 mL distilled water. The flasks were incubated at 30°C and 200 rpm for 24 h, to reach an optical density close to 4.0 at 600 ηm (OD_{600}).

Table 10. The composition of the Hutner solution.

Hutner Solution	
Nitrilotriacetic acid	2.5 g
$CaCl_2 \cdot 2H_2O$	1.23 mg
$FeSO_4 \cdot 1,5H_2O$	19.6 mg
$Na_2MoO_4 \cdot 2H_2O$	3.9 mg
Metallic solution	13.9 mL
Water	q.s.p. 250 mL
pH	7.2
Metallic solution	
Na_2EDTA	250 mg
$ZnSO_4 \cdot 7H_2O$	680 mg
$FeSO_4 \cdot 7H_2O$	356 mg
$MnSO_4 \cdot H_2O$	154 mg
$CuSO_4 \cdot 5H_2O$	39.2 mg
$CoCl_2 \cdot 6H_2O$	20.3 mg
$Na_2B_4O_7 \cdot 10H_2O$	17.7 mg
Water	q.s.p. 100 mL

2.3. Cell cultures

Twenty milliliters of the pre-culture were aseptically transferred to a 500 mL conical flask with the same culture medium as described above. However, the sole carbon source consisted of 0.5 g of one of the terpene substrates diluted in 12.5 mL *n*-hexadecane. The flasks were left at 30°C and 200 rpm for 24 h.

2.4. Recovery of the biomass

After centrifuging the culture at 2,600 *g* for 10 min, the supernatant was eliminated and the resulting biomass was resuspended in 25 mL 20 mM phosphate buffer pH 7.5. This biomass was either used directly (biotransformation with fresh cells) or was frozen (-18°C) for the trials using crude enzymatic extracts. The biomass concentration was determined indirectly by the OD_{600} of the medium, through the following experimental correlations: *P. rhodesiae* (FONTANILLE *et al*. 2002): biomass (g per liter of medium) = 0.41 x OD_{600}; *P. flourescens*: biomass (g per liter of medium) = 0.35 x OD_{600}.

2.5. Production of the crude enzymatic extracts

The concentrated biomass of *P. rhodesiae* (see preceding paragraph) was thawed and treated for 1 h at 30°C and 200 rpm with 6% (v/v) diethyl ether (FONTANILLE; LARROCHE 2003). The *P. fluorescens* strain employed in this study has shown a relatively high resistance to cell permeabilization, in comparison to *P. rhodesiae.* Hence, the standard protocol used for this last microorganism allowed 70-90% of the initial biomass to remain viable. The cells were then disrupted by a 3 cycle passage in a two-stage high-pressure homogenizer (APV Systems, Alberstlund, Denmark, model APV 2000), using 1,000 bars in the first stage and 100 bars in the second. This methodology was able to reduce the final number of viable cell counts to 5% of the

initial value. The suspension resulting from this operation was membrane filtered (0.45µm) to remove the remaining viable cells.

2.6. Biotransformation procedure

Twenty-five milliliters of the concentrated biomass and the same volume of *n*-hexadecane were transferred to a 250 mL conical flask. The substrate was added to reach a final concentration of 40 g per liter of *n*-hexadecane. The flasks were incubated at 30°C and 200 rpm. Samples were periodically taken from the organic and aqueous phases to follow substrate consumption and product formation. The organic phase was directly injected (1 µL) in the gas chromatograph while the aqueous phase had to be acidified (20 $\mu L.mL^{-1}$ concentrated sulfuric acid) and extracted with the same volume of ether-hexane (1:1, v/v) before the organic layer was injected (1 µL) in the gas chromatograph. The procedure for the crude enzymatic extract was similar.

2.7. Analytical conditions

The products obtained were analyzed in a HP 5890 gas chromatograph with a flame ionization detector (GC-FID). A SBP-5 (Supelco) capillary column of 30 m x 0.32 mm x 0.25 µm id was employed. Nitrogen was the carrier gas, with a constant pressure in the head of the column of 0.8 bar and split ratio of 1:5. The temperature program used was as follows: initial temperature of 80 °C for 5 min, rising at 20 $°C.min^{-1}$ until 200 °C, then held for 5 min. The temperatures in the injector and detector were both 250 °C. The quantification was performed using 10 mL heptadecane (Fluka, ≥98% purity) per liter of *n*-hexadecane as internal standard and the yield was calculated as the ratio of the amount of product recovered (g) to the mass of substrate consumed (g). The products obtained were identified by a HP 6890 gas chromatograph coupled with a HP 5973 mass selective detector (GC-MS). Helium was the carrier gas and the

split ratio was 1:5. The capillary column and the temperature program was the same as above. The MS system operated with an electron impact of 70eV, an acceleration voltage of 1.1kV and an emission current of 35μA. The temperatures of the quadrupole, the ionic source and the GC-MS interface were 150 °C, 230 °C and 280 °C, respectively. The isomers obtained, *i.e.* novalal and isonovalal, were identified on the basis of their retention times and mass spectra. An example of a GC chromatogram is shown in Figure 21.

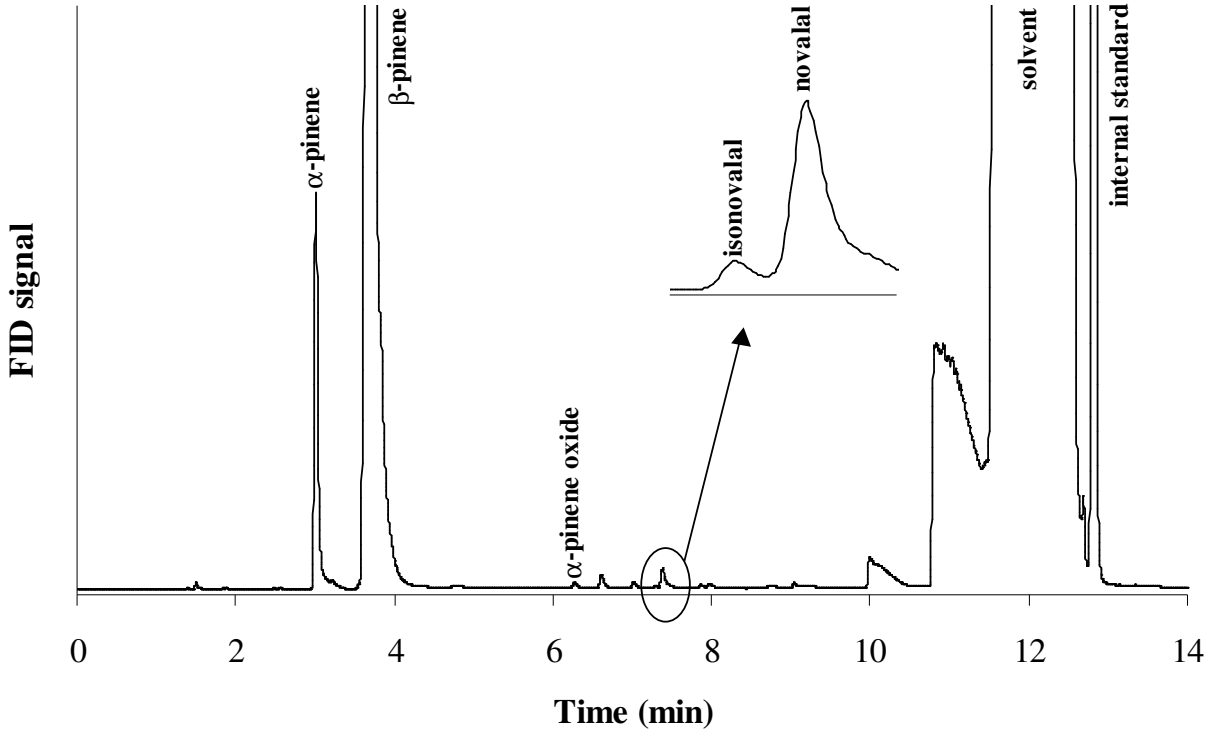


Figure 21. Example of a CG chromatogram: The organic phase of bioconversion of β-pinene by fresh cells of *P. rhodesiae* grown on β-pinene. Experimental conditions described in Table 13.

3. Results

3.1. Characterization of the complex substrates

Turpentine and orange peel oil are cheap and widespread terpene sources and could be involved in bioconversion processes, either as inducing agents for biocatalyst production or as precursors for the reaction itself.

As for any other agroindustrial product, their composition may vary depending on seasonal conditions. The composition of turpentine and orange peel oil, shown in Table 11, was determined by GC-FID and GC-MS. The concentration of the main compound in each mixture was consistent with reported values (Table 11).

Table 11. Main constituents of turpentine and orange peel oil used in this study.

Component	**Proportion (%, area in GC)**	**Reported values (%)**
Turpentine		
α-Pinene	67.1	60-70 (BAUER *et al*., 2001); 43-89 (COPPEN *et al*., 1998)
Camphene	20.6	
R-(+)-limonene	4.6	
others	7.7	
Orange peel oil		
R-(+)-limonene	87.7	83-97 (SHAW, 1979); 94.3 (MARÓSTICA; PASTORE 2007b)
cis and *trans* limonene-1,2-oxide	3.6	
Carvone	1.6	
others	7.1	

3.2. Utilization of the terpene substrates for bacterial growth

The two microorganisms were first tested for their ability to grow on terpene substrates as sole carbon and energy sources. The growth was stopped after 24 h because the biomass obtained at this time was a good indicator of the ability of a given substrate to support bacterial growth. The utilization of a given terpene indicated that a metabolic pathway involving it was actually active in the corresponding bacterial strain.

It can be observed in Table 12 that α-pinene, β-pinene and α-pinene oxide were substrates for both microorganisms. As expected, turpentine was also a substrate for the two bacteria, probably operating as an α-pinene source. The other constituents of turpentine were not toxic to the bacteria, at least at the concentration assayed. Limonene and β-pinene oxide were substrates only for *P. fluorescens*, while limonene-1,2-oxide and orange peel oil were substrates for none of the two strains. All the degradable terpene substrates produced biomass with concentrations ranging from 0.3-1.8 $g.L^{-1}$ and these were usually lower for the epoxides (Table 12).

Table 12. Utilization of the terpene substrates by *P. rhodesiae* and *P. fluorescens* for growth. The inoculum consisted of a culture grown on a medium with 0.25 g $(NH)_4SO_4$, 5 mL Hutner solution (Table 10), 10 mL solution A (6.5 g K_2HPO_4, 8.28 g KH_2PO_4 in 250 mL distilled water), 1 g Na-lactate (*P. rhodeisae*) or 1 g glucose (*P. fluorescens*) and 235 mL distilled water. Cell growth was developed in the same medium using 0.5 g of the terpene substrate in 12.5 mL *n*-hexadecane as sole carbon source*.

Terpene substrate	Growth after 24h	
	P. rhodesiae	*P. fluorescens*
α-pin	+ (0.9)	+ (1.7)
β-pin	+ (1.8)	+ (1.6)
Lim	–	+ (1.4)
α-pin ox	+ (0.3)	+ (0.8)
β-pin ox	–	+ (0.9)
Lim ox	–	–
Turp	+ (1.1)	+ (1.8)
OPO	–	–

* α-pin: α-pinene; β-pin: (–)-β-pinene; Lim: *R*-(+)-limonene; α-pin ox: (–)-α-pinene oxide; β-pin ox: β-pinene oxide; Limo ox: (+)-limonene-1,2-oxide; Turp: turpentine; OPO: Orange peel oil; +: growth; –: no growth. Numbers in brackets are the approximate final biomass concentration ($g.L^{-1}$).

3.3. Biocatalytic activity of *P. rhodesiae* and *P. fluorescens*

To define the metabolic pathways through which the terpene substrates were degraded and to find activities that could be exploited at a preparative scale, bioconversion experiments were carried out with either concentrated fresh cells or crude enzymatic extracts. The latter could be considered as a form of the biocatalyst unable to perform cofactor-dependent reactions. Results obtained are presented in Table 13, with the main products accumulated after 24h of bioconversion and their respective approximate yields. When product accumulation and substrate consumption were very low, the parameter "amount produced" was chosen as a more reliable option.

Table 13. Main products accumulated in the bioconversion of the α-pinene, β-pinene and limonene families by *P. rhodesiae* and *P. fluorescens*. The culture grown on a terpene substrate as sole carbon source (24 h at 30°C and 200 rpm) was concentrated ten times and the resulting biomass was either used fresh or as a crude enzymatic extract (1 h at 30°C and 200 rpm, in the presence of 6% ether, for *P. rhodesiae*, or three cycle passage in a two-stage APV Systems homogenizer, using 1,000 bar in the first stage and 100 bar in the second, for *P. fluorescens*, before being used). The bioconversion trials consisted of a 250 mL conical flask filled with 25 mL concentrated biomass, 25 mL *n*-hexadecane and 1g terpene substrate incubated for 24 h at 30°C and 200 rpm [*].

Terpene substrates		Products accumulated after 24h			
Cell growth	Bioconversion	*P. rhodesiae*		*P. fluorescens*	
		Fresh	Crude enzymatic extract	Fresh	Crude enzymatic extract
α-pin	α-pin	DMPA (10)	0	DMPA (2)	0
α-pin	α-pin ox	isonov (6), nov (11), nov ac (74)	isonov (80[‡])	nov (47), nov ac (18)	isonov (66[‡])
α-pin ox	α-pin ox	isonov (46), nov (7), nov ac (2)	–	nov (10), nov ac (17)	–
Turp	Turp[†]	DMPA (8)	0	α-pin ox (3), nov (6)	0
Turp	α-pin ox	isonov (13), nov (24), nov ac (35)	isonov (75[‡])	–	–
β-pin	β-pin	α-pin (14), DMPA (7)	α-pin (0.4[§])	α-pin (20), DMPA (1)	α-pin (0.3[§])
β-pin	β-pin ox	autodegrad	autodegrad	autodegrad	Autodegrad
β-pin ox	β-pin ox	**	**	autodegrad	Autodegrad
Lim	Lim	**	**	A-ter (49)	α-ter (91)
Lim	OPO[††]	**	**	[‡‡]	α-ter (0.5[§]), Lim diol (0.2[§])
Lim	Lim ox	**	**	Lim diol (27), HOL (77)	Lim diol (0.5[§])

* 0: no products; –: not done; α-pin: α-pinene; α-pin ox: α-pinene oxide; α-ter: α-terpineol; β-pin: (–)-β-pinene; β-pin ox: β-pinene oxide; autodegrad: the same products obtained in the control experiments (inactive biomass); DMPA: 3,4-dimethyl pentanoic acid; HOL: 1-hydroxy-2-oxolimonene; isonov: isonovalal; Lim: *R*-(+)-limonene; Lim diol: limonene-1,2-diol; Lim ox: limonene-1,2-oxide; nov: novalal; nov ac: novalic acid; OPO: Orange peel oil; Turp: turpentine. Numbers in brackets are the approximate yield (%). † only α-pinene is metabolized in turpentine, while the other constituents are not consumed. ‡ after 6h bioconversion. § Product concentration ($g.L^{-1}$) after 24h (too low substrate consumption and product formation to calculate a reliable yield). ** No growth on the carbon source. ††Limonene and part of limonene-1,2-oxide are metabolized in orange peel oil. ‡‡ Orange peel oil permeabilizes the biomass.

3.3.1. Alpha and β-pinene

The results shown in Table 13 indicate that both precursors were consumed and all the products accumulated by both strains were basically from the same metabolic pathway (pathway 1b in Figure 19). When a fresh biomass of *P. rhodesiae* grown on α-pinene was used to convert α-pinene, 3,4-dimethyl pentanoic acid (DMPA) was the major product accumulated after 24h. The same feature was observed for fresh *P. fluorescens* cells, but with lower yields. Alpha-pinene oxide, the first intermediate in α-pinene degradation, allowed the accumulation of isonovalal, novalal, novalic acid and traces of DMPA with *P. rhodesiae*, while isonovalal could not be detected with *P. fluorescens*. The behavior of the crude enzymatic extracts (Table 13) demonstrated that, for both strains, epoxidation of α-pinene was a cofactor-dependent process, while aldehyde synthesis from the epoxide was cofactor independent.

The bioconversion of β-pinene by fresh cells of *P. rhodesiae* and *P. fluorescens* preliminarily grown on the same carbon source resulted in the formation of α-pinene, DMPA and

traces of intermediates shown in pathway 1b in Figure 19 (Figure 21). The same reaction with the crude enzymatic extracts of both strains gave only a low α-pinene accumulation, not observed in the control experiments (Table 13).

When β-pinene oxide was used as substrate, the products obtained were always the same as those found in the control experiments (without biomass). This behavior was evidenced for all kinds of biomass and strains used in this work (Table 13). It was thus concluded that the main phenomenon was a precursor autooxidation in the aqueous phase, which took place without any significant biocatalytic activity.

3.3.2. Limonene

P. rhodesiae did not grow on limonene as sole carbon source, it was thus not considered for the limonene bioconversion trials shown in Table 13.

For the fresh cells and crude enzymatic extract of *P. fluorescens* biomass, the sole metabolite accumulated was α-terpineol, the yield being higher with the extract. The production obtained in this process, around 10-11 g per liter of *n*-hexadecane after 30 h - 40 h at a maximum rate of ~ 1 $g.L^{-1}.h^{-1}$ (Figure 22), was in fact, to the best of our knowledge, the highest already reported for the bioproduction of α-terpineol.

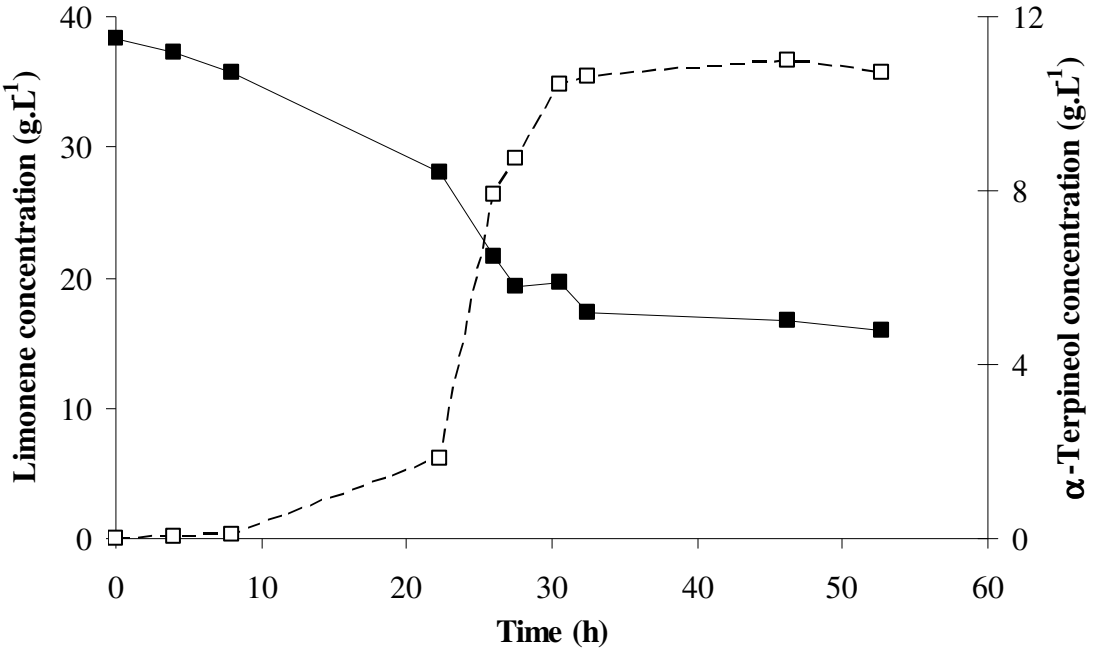


Figure 22. Bioconversion of limonene (■) to α-terpineol (□) using fresh cells of *P. fluorescens* grown on limonene as sole carbon source. The experimental conditions as described in Table 13.

Interestingly, it was noticed that whole cells of this microorganism were also able to accumulate limonene-1,2-diol and 1-hydroxy-2-oxolimonene from limonene-1,2-oxide, a process practically inoperative with the crude extract (Table 13).

3.3.3. Turpentine and orange peel oil

The bioconversion of turpentine (source of α-pinene) by fresh *P. rhodesiae* cells grown on the same substrate gave almost the same profile as that observed for the conversion of pure α-pinene. This result demonstrated that this essential oil was also able to act as a cell inducer for α-pinene degradation. The other major constituents of turpentine (camphene, limonene) were never

metabolized (Table 13). The substitution of α-pinene by turpentine for the cell growth in the process of isonovalal production (FONTANILLE; LARROCHE, 2003) resulted in similar results to that obtained in the standard process, *i.e.*, maximal concentration of ~ 80 $g.L^{-1}$ obtained after 4h, with an initial maximum rate of ~ 40 $g.L^{-1}.h^{-1}$ and a yield of 75-80 % (Figure 23).

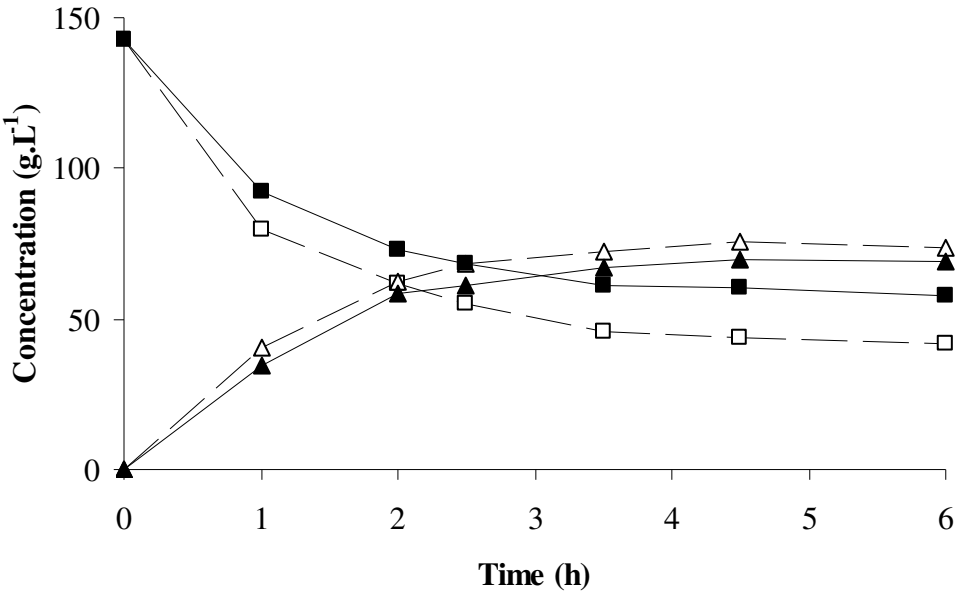


Figure 23. Bioconversion of α-pinene oxide (squares) to isonovalal (triangles) using permeabilized cells of *P. rhodesiae* induced by α-pinene (black symbols, continuous lines) or turpentine (white symbols, dash lines) during the cell growth. Five milliliters of α-pinene oxide was used as substrate. The other experimental conditions as described in Table 13.

It was not possible to perform the bioconversion of orange peel oil with fresh *P. fluorescens* biomass because, after a short period (<6 h), the cells were dead, as evidenced by the absence of growth on the agar medium. The biocatalyst could thus be considered as permeabilized and thus behaving like a crude enzymatic extract after this period, which might

explain why the microorganism grew on limonene but not on orange peel oil (~88% limonene) as sole carbon source (Table 12). Moreover, the limonene biotransformation activity using orange peel oil as substrate appeared dramatically lower than that achieved with pure limonene (Table 13). It was thus assumed that orange peel oil, in addition to its inactivating action, also acted as an enzyme inhibitor. Trials with mixtures of *R*-(+)-limonene, limonene-1,2-oxide and carvone in the same concentrations as found in orange peel oil indicated that the growth-inhibition agent was not one of the main constituents of the oil and that limonene-1,2-oxide was the main compound responsible for the enzymatic inhibitory effect (data not shown). More detailed studies are however still needed to get a better understanding of the inhibition phenomenon due to orange peel oil.

4. Discussion

In accordance with previous results (BEST *et al*., 1987; FONTANILLE; LE FLÈCHE; LARROCHE, 2002), the metabolism of α-pinene by *Pseudomonas rhodesiae* and *P. fluorescens* was found to be basically the same (pathway 1b in Figure 19), although some discrepancies in the level of enzymatic activities were noticed between them. For example, in contrast with the well known feature of *P. rhodesiae*, the fresh cells of *P. fluorescens* did not accumulate isonovalal during the bioconversion of α-pinene oxide. Also, α-pinene and its metabolites seemed to be almost completely metabolized by this strain after 24h, while the fresh biomass of *P. rhodesiae* accumulated DMPA with a significant yield.

It was also noticed that the carbon source used for biomass production acted as an inducing agent and had a major effect on the yields and rates achieved during the bioconversion phase. For example, the use of α-pinene oxide for cell growth considerably increased isonovalal accumulation in fresh *P. rhodesiae* and lowered novalal accumulation in whole cells of *P.*

fluorescens, in comparison to data observed with α-pinene used as carbon source. It was demonstrated that turpentine (source of α-pinene) could be used as carbon source for biocatalyst production for further bioconversion of α-pinene oxide. This feature opened a possibility to lower the cost of biocatalyst production.

Epoxidation of α-pinene did not occur in the presence of the crude enzymatic extracts of either *P. rhodesiae* or *P. fluorescens*, which confirmed that this conversion was carried out by a NADH-dependent monooxygenase (BEST *et al*., 1987). As no β-pinene oxide was formed in the bioconversion of β-pinene, it was assumed that this enzyme had narrow substrate specificity, just like the following enzyme in the pathway, α-pinene oxide lyase.

The results obtained for β-pinene revealed that this compound could be metabolized by both strains used in this study. This result was, to our knowledge, observed for the first time. It was supposed that this compound was metabolized by a cofactor independent isomerase via α-pinene as described in pathway 1b in Figure 19, since the pathway reported by Yoo and Day (2002) involved limonene as intermediate, which was never detected in our experiments.

The biotransformation of limonene to α-terpineol (pathway 4 in Figure 20) has already been reported and studied for many other microorganisms (ADAMS; DEMYTTENAERE; KIMPE, 2003; KRAIDMAN; MUKHERJEE; HILL, 1969; MARÓSTICA; PASTORE 2007b; TAN; DAY 1998a,b; TAN; DAY; CADWALLADER, 1998), including a *Pseudomonas* species (CADWALLADER *et al.*, 1989), although not for *P. fluorescens* NCIMB 11671. The results obtained with the crude enzymatic extracts in this work have shown that this reaction was catalyzed by a cofactor-independent enzyme, similar to α-terpineol dehydratase from *P. gladioli* (CADWALLADER; BRADDOCK; PARISH, 1992). Also, as for *Fusarium oxysporum* (MARÓSTICA JR.; PASTORE, 2007b), the enzyme responsible for this conversion seemed non inducible, because α-terpineol was still produced by a biocatalyst grown on glucose (data not

shown). The lack of α-terpineol production during the bioconversion of turpentine by *P. fluorescens* might be explained by a catabolic repression of α-pinene in limonene biotransformation (YUSTE; CANOSA; ROJO, 1998).

The accumulation of limonene-1,2-diol and 1-hydroxy-2-oxolimonene during the limonene-1,2-oxide bioconversion led to suggest that *P. fluorescens* might have another pathway for limonene, similar to that described by van der Werf *et al*. (1999) (pathway 2 in Figure 20). The first enzyme of this metabolic route, limonene-1,2-epoxide hydrolase was cofactor independent, but the following steps necessarily needed cofactors and, thus, could be performed only by aerobically operated whole cells. It was thus proposed that the two limonene metabolic routes took place simultaneously in whole *P. fluorescens* cells (pathways 2 and 4 in Figure 20). The first, passing through limonene-1,2-oxide, was an aerobic process leading to energy production, while the second, leading to α-terpineol, was oxygen independent and could be viewed as a process for detoxification of the medium (BAKKALI *et al.* 2007; BICAS; PASTORE 2007; GRIFFIN *et al.*, 1999).

5. Conclusion

The use of whole cells and crude enzymatic extracts was an original approach of this study. It was designed to identify parts of pathways that could be considered as cofactor independent, *i.e.*, that could be carried out without aeration of the medium.

Results achieved in this study demonstrated that *P. rhodesiae* could be considered as a specialist for the degradation of the α- and β-pinene family through the aldehyde (pathway 1b in Figure 19).

P. fluorescens, on the other hand, appeared to be a more versatile microorganism. Besides degrading α- and β-pinene in the same way as *P. rhodesiae,* this strain also metabolized limonene through two pathways, the most efficient being the synthesis of α-terpineol.

Results obtained with complex terpene mixtures confirmed that the toxicity of these compounds is highly variable. Turpentine did not contain inhibitors for the two strains used in this study and could thus be used as an α-pinene source. It was not the case for orange peel oil, which could not be used as a limonene supplier.

CAPÍTULO 6

METHOD FOR THE PRODUCTION OF INCREASED CONCENTRATIONS OF *R*-(+)-α-TERPINEOL BY THE BIOTRANSFORMATION OF *R*-(+)-LIMONENE

1. Introduction

As already stated in the other chapters, the biotechnological conversions of *R*-(+)-limonene for the production of bioflavors have been considered in the past few years. However, the low yields and high production costs usually discourage their commercial adoption. α-Terpineol, one of its oxygenated derivative, is a stable alcohol typically applied in household products, cosmetics, pesticide and flavor preparations (BAUER; GARBE. SURBURG, 2001; SINGH *et al*. 1998). This monoterpenoid is an important commercial product commonly produced by acid-catalyzed chemical synthesis from α-pinene or turpentine oil. Nevertheless, the biotransformation of limonene to α-terpineol as main product has already been described for the fungi *Cladosporium* sp. (KRAIDMAN. MUKHERJEE; HILL, 1969), *Penicillium digitatum* (TAN; DAY 1998a,b; TAN; DAY; CADWALLADER, 1998; ADAMS; DEMYTTENAERE; DE KIMPE, 2003; DEMYTTENAERE; VAN BELLEGHEM; DE KIMPE, 2001) and *Fusarium oxysporum* (MAROSTICA JR.; PASTORE 2007b, BICAS *et al*. 2008a). For bacteria, this conversion pathway has been reported in *Pseudmonas gladioli* (CADWALLADER *et al*. 1989; CADWALLADER; BRADDOCK; PARISH, 1992), a recombinant *E. coli* (SAVITHIRY; CHEONG; ORIEL, 1997) and *P. fluorescens* (BICAS *et al*. 2008b). The bioproduction of α-

terpineol as main product has also been shown as possible using α- and β-pinenes as substrates (CHATTERJEE; DE; BHATTACHARYYA, 1999; ROZENBAUM *et al*. 2006; TONIAZZO *et al*. 2005; WRIGHT *et al*. 1986).

The main drawback of monoterpenes biotransformation processes are the chemical instability, high volatility and high cytotoxicity of both precursors and products, the low solute solubility, and the low transformation rates (KRINGS; BERGER, 1998). The use of biphasic systems has been demonstrated to be an efficient technique since it eases the product recovery and increases yields by reducing the substrate and product toxicity and their losses by volatilization (CABRAL, 2001). The organic phase is usually hydrocarbon solvents (*n*-decane, *n*-hexadecane) with an octanol:water partition coefficient (log P_{ow}) higher than 4, a commonly accepted requisite for a good tolerance by whole microbial cells (CABRAL, 2001). This strategy has already been applied for the production of isonovalal from α-pinene oxide by *P. rhodesiae* (FONTANILLE; LARROCHE 2003) and patented for the bioconversion of some terpenes (MULLER *et al*., 2007). The use of vegetable oils as organic phase, already used for the biotransformation of sterols (STEFANOV; YANKOV; BESCHKOV, 2006), has not, up to now, been considered for the bioconversion of monoterpenes, although they could be considered as an environment friendly alternative to organic solvents.

Production of 0.7 $g.L^{-1}$ (KRAIDMAN; MUKHERJEE; HILL, 1969), 1 $g.L^{-1}$ (CADWALLADER *et al*., 1989) and 2.4 $g.L^{-1}$ (BICAS *et al*., 2008a) of α-terpineol from limonene have already been reported, while the highest recovery of α-terpineol reported so far, about 3.2 $g.L^{-1}$, has been obtained using sequential substrate feedings (TAN; DAY; CADWALLADER, 1998). However, these values appear to be too low to allow an economic development of these processes. In this manuscript, a methodology for the production of high

amounts of *R*-(+)-α-terpineol from *R*-(+)-limonene in biphasic medium using vegetable oils as organic phase is reported. It seems to be the most efficient alternative for the commercial production of natural *R*-(+)-α-terpineol reported so far.

2. Materials and Methods

2.1. Microorganisms and chemicals

The strain employed in this work, formerly known as *Pseudomonas fluorescens* NCIMB 11671, has been re-identified as *Sphingobium* sp. The terpene compounds *R*-(+)-limonene (Fluka, ~98% purity), *S*-(–)-limonene (Fluka, ≥95% purity), (+)-limonene-1,2-oxide (Aldrich, mixture of cis/trans isomers, 97% purity), (+)-carvone (Acros Organics, 98% purity), *R*-(+)-α-terpineol (Fluka, ~99% purity) and α-terpineol (Aldrich, ~98% purity, $[\alpha]^{19} = -30°$) were kept under refrigeration temperature. *n*-Hexadecane (SDS, 99% purity), sunflower oil (commercial grade), rapeseed oil (commercial grade), heptadecane (Fluka, ≥98% purity) and 1-pentanol (Acros Organics, 99% purity) were kept at ambient temperature.

2.2. Biocatalyst production

Three full loops of a 24 h old culture on Petri dish were transferred to a 500 mL conical flask containing 1.0 g glucose, 0.25 g $(NH)_4SO_4$, 5 mL Hutner solution (Table 10), 10 mL of solution A and 235 mL distilled water. Solution A was made of 6.5 g K_2HPO_4 and 8.28 g KH_2PO_4 in 250 mL distilled water. The flasks were incubated at 30°C and 200 rpm for 24 h, time at which the optical density at 600 ηm (OD_{600}) was close to 4.0. The resulting cultivation medium was referred to as pre-culture.

Twenty milliliters of the pre-culture were aseptically transferred to a 500 mL conical flask with the same culture medium as described above except that glucose was replaced by an organic solution containing 500 µg of limonene in 12.5 mL of *n*-hexadecane (final concentration of 40 g per liter of organic phase). The flasks were left at 30°C and 200 rpm for 24 h, time at which the OD_{600} of the aqueous medium reached 0.8.

Growth experiments in the bioreactor (4.8 L working volume) were performed using 170 mL of the above pre-culture as inoculum, 4 L distilled water, 170 mL solution A, 85 mL Hutner solution, 4.25 g $(NH)_4SO_4$, 212.5 mL hexadecane and 15 g limonene (70 g/L organic solvent). Temperature, agitation and aeration were kept at 30°C, 500 rpm and 0.3 to 0.5 slpm, respectively. The CO_2 and O_2 in the air exiting from the bioreactor were monitored (Servomex 4100 Gas Purity Analyzer), the pH was controlled at 6, the OD_{600} of the liquid phase and the composition of organic and aqueous phases (GC-FID, see item 2.4) were monitored.

After centrifuging the culture medium at 2,600*g* for 10 min, the resulting biomass was resuspended in phosphate buffer 20mM pH 7.5 in order to achieve a ten-fold concentration. This biomass was either used directly for the biotransformation with fresh cells (biomass from conical flasks) or frozen (-18°C) before its use (biomass from bioreactor). The biomass concentration was determined using the formula (BICAS *et al.*, 2008b):

$$\text{Biomass } (g.L^{-1} \text{ medium}) = 0.35 \times OD_{600}.$$

2.3. Biotransformation procedure

Twenty five milliliters of the concentrated biomass (OD_{600} = 7 for fresh cells and OD_{600} = 13 for frozen and thawed biomass) and the same volume of organic phase were transferred to a 250mL conical flask. The substrate was added to reach a final concentration of 40 g per liter of organic phase. The flasks were incubated at 30°C and 200 rpm. Anaerobic bioconversion

involved the use of 250 mL conical flasks with two entries (one at the base and another at the neck) plugged with a rubber stopper instead of polyurethane plugs. At the beginning of the conversion, as well as after each sampling, the medium was flushed by bubbling N_2 (~ 0.5 $L.min^{-1}$) from the base entry for 5 min. For the bioconversion in bioreactor (0.5 L), 150 mL of concentrated frozen cells and the same volume of organic phase with 40 $g.L^{-1}$ of *R*-(+)-limonene were maintained at 30 °C and 800 rpm.

Samples were periodically taken from the organic phase to follow the substrate consumption and the formation of products. When *n*-hexadecane was used as organic phase, it was directly injected (1 µL) in the gas chromatograph while sunflower oil had to be extracted (1 min vortexing) with the same volume of 96 vol % ethanol which was injected (1 µL) in the gas chromatograph.

2.4. Analytical conditions

The products obtained were analyzed in a HP 5890 gas chromatograph with flame ionization detector (GC-FID). A SBP-5 (Supelco) capillary column of 30 m x 0.32 mm i.d. x 0.25 µm film thickness was employed. Nitrogen was used as gas carrier with a constant pressure at the head of the column of 0.8 bar and the injection split ratio was 1:5. The oven temperature program involved an initial temperature of 80°C hold for 5min, then rise at 20 $°C.min^{-1}$ to 200 °C, value maintained for 5min. The injector and detector temperatures were both 250 °C. Solute quantification was performed after adding 1% ($v.v^{-1}$) heptadecane in *n*-hexadenane samples or 0.2% ($v.v^{-1}$) 1-pentanol in ethanol samples as internal standards and concentrations were expressed as mass of product per liter of organic phase.

The identification of enantiomeres was carried out by comparison the retention time of standards (*R*-(+)-α-terpineol and α-terpineol ($[\alpha]^{19} = –30°$; majority of *S* isomer)) with samples. A

Beta Dex™ 120 fused silica capillary column (Supelco; 30m, 0.25mm i.d., 0.25µm film thickness) was mounted in a HP 6890 gas chromatograph with flame ionization detector (GC-FID) with an oven temperature maintained at 120 °C for 20 min, then rise to 200 °C at 50 ºC.min^{-1}, value hold for 5min. The other conditions were the same as described above. The enantiomeric excess (ee) was estimated by the ratio of the R/S areas as obtained after GC analysis.

Unknown products were analyzed by a HP 6890 gas chromatograph coupled to a HP 5973 mass selective detector (GC-MS). The carrier gas was helium and the injection split ratio was 1:5. The capillary column was the already mentionned SBP-5 used with the same conditions as above. The MS system was operated with an electron impact of 70 eV, an acceleration tension of 1.1 kV and an emission current of 35 µA. The temperatures of quadrupole, ionic source and interface were 150 °C, 230 °C and 280 °C, respectively.

3. Results and Discussion

3.1. Limonene as sole carbon source for the bacterial growth

The culture of *Sphingobium* sp. with limonene as sole carbon source was monitored in a 4.8 L bioreactor coupled with a gas purity analyzer (see item 2.2). The aeration varied depending on the microorganism's oxygen uptake. The results obtained are shown in Figures 1 and 2. It is possible to observe that the exponential growing phase (μ_{max} = 58 mg.L^{-1}.h^{-1}), allied to an exponential oxygen uptake and CO_2 formation, began after 25 h to 30 h and it was extended till 40 h. About 8 h later, the entire nitrogen source was consumed and a small decrease of yCO_2 together with an increase of both yO_2 and pH was noticed, indicating the end of the culture. At this stage, the culture presented a concentration of 1.4 g of biomass per liter of aqueous phase (OD_{600} = 4). The global biomass yield was 67% (m.m^{-1}).

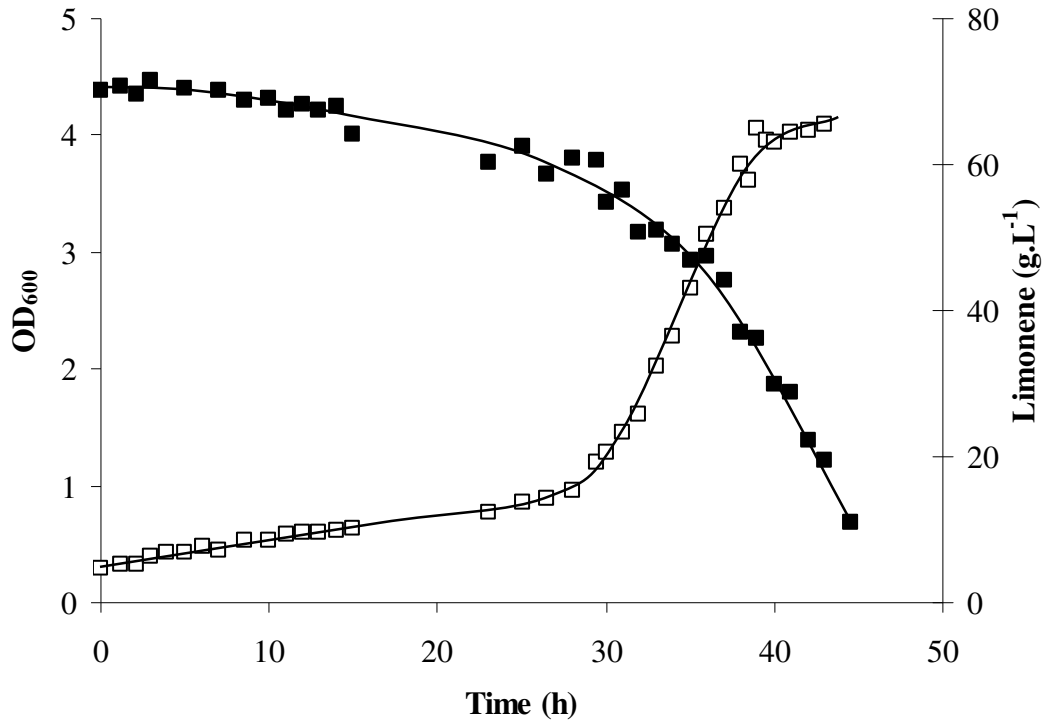


Figure 24. Growing curve of *Sphingobium* sp. (□) in *R*-(+)-limonene (■) as sole carbon source. Culture developed in a 4.8L bioreactor at 30°C/500rpm and aeration of 0.3-0.5slpm using *n*-hexadecane as organic phase.

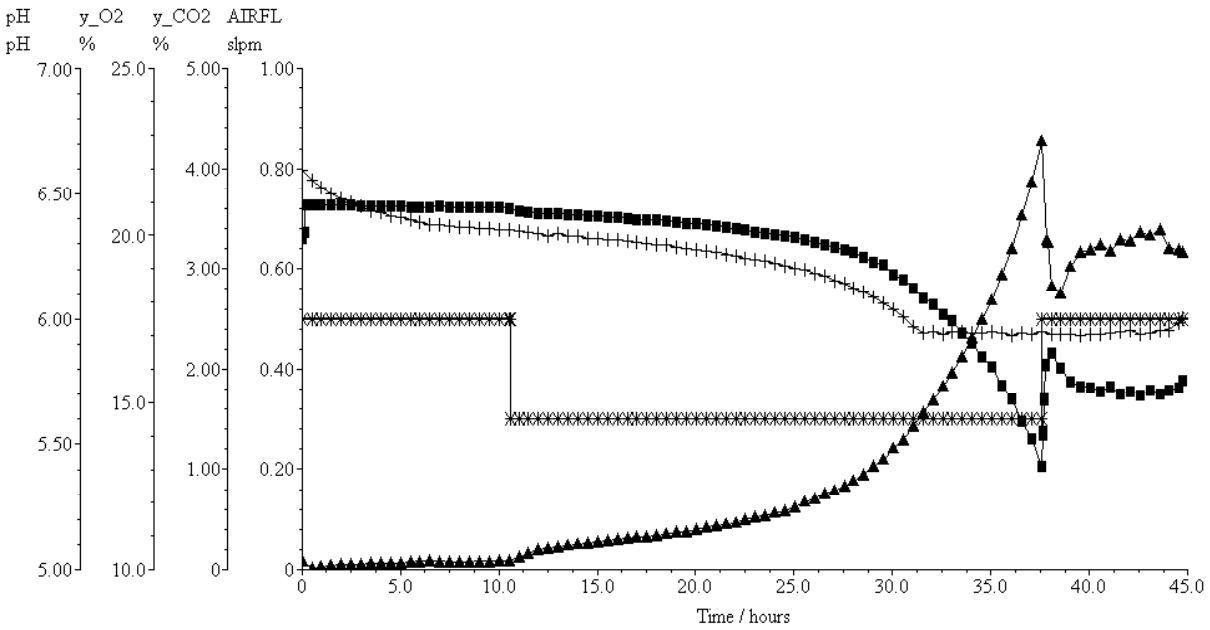


Figure 25. Aeration (**x**), pH (**+**), O_2 uptake (■) and CO_2 production (▲) in the culture of *Sphingobium* sp. in *R*-(+)-limonene as sole carbon source. Culture conditions described in Figure 24.

It is worth noticing that traces of limonene-1,2-diol were present during all the growing phase (<38 h) and that α-terpineol was accumulated in low amounts between 38 h to 44 h (end of exponential phase - beginning of stationary phase) (data not shown). These observations reinforces the hypothesis that this microorganism presents two parallel metabolisms for limonene: one that passes through limonene-1,2-diol to produce energy and another secondary metabolism devoted to limonene detoxification by the formation of α-terpineol (BICAS *et al*., 2008b).

3.2. Enantioselectivity of the enzyme

The trials with *R*-(+)- and *S*-(–)-limonene showed that the enzyme responsible for the conversion of these substrates to α-terpineol was enantiospecific. When *R*-(+)-limonene was used as substrate, *R*-(+)-α-terpineol was produced (ee ≥99%) and the *S*-(–)-isomer gave *S*-(–)-α-terpineol (ee ~ 80%). Interestingly, there was a higher latency phase in the bioconversion of *S*-(–)-limonene, that might indicate that this strain was more adapted to the *R*-(+)-limonene/*R*-(+)-α-terpineol biotransformation and it needed a period of adaptation to be able to convert the *S*-(–)-isomer. However, apart from this retarded beginning, the bioconversion profile was basically the same for both isomers (Figure 26). The bioconversion yield, maximal conversion rate and maximal concentration were, respectively, 75-85%, ~ 0.2 $g.L^{-1}.h^{-1}$ organic phase and 10-11 $g.L^{-1}$ organic phase in both cases. *Penicillium digitatum* is able to promote only the *R*-(+) to *R*-(+) conversion (TAN; DAY; CADWALLADER, 1998; ADAMS; DEMYTTENAERE; DE KIMPE, 2003), while *Pseudomonas gladioli* converts enantiospecifically both limonene isomers, although the rate of hydroxylation of *R*-(+)-limonene to *R*-(+)-α-terpineol is almost ten times higher when compared to the conversion of *S*-(–)-limonene to *S*-(–)-α-terpineol (BRADDOCK; CADWALLADER, 1995).

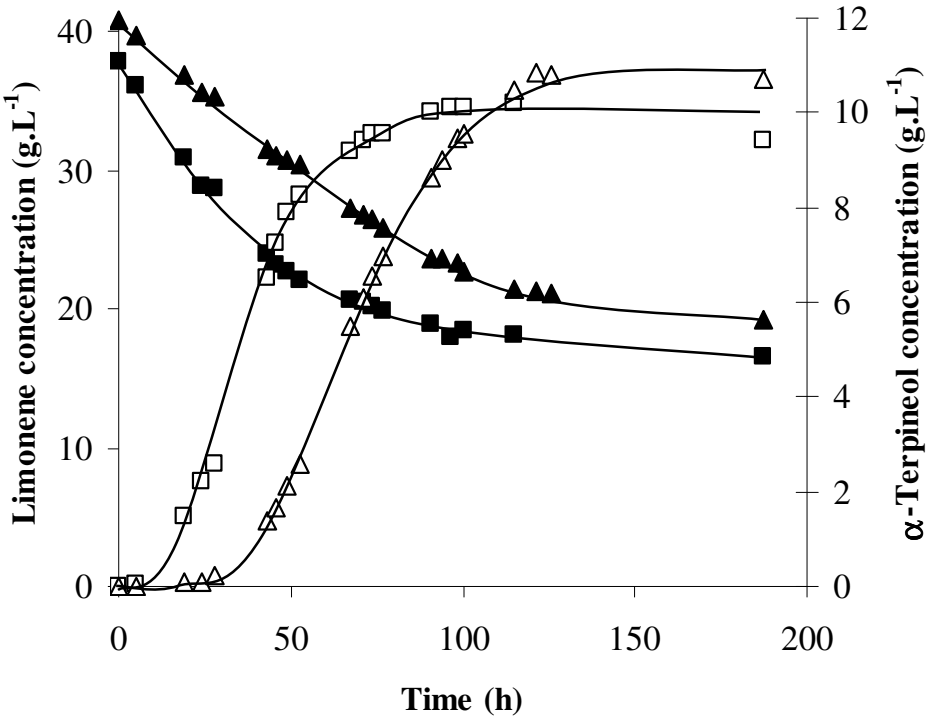


Figure 26. Bioconversion of *R*-(+)-limonene (■) and *S*-(–)-limonene (▲) to, respectively, *R*-(+)-α-terpineol (□) and *S*-(–)-α-terpineol (△) by fresh *Sphyngobium* sp. using *n*-hexadecane as organic phase.

3.3. Inducibility of the enzyme

As may be observed, there were no significant differences between the biotransformation of *R*-(+)-limonene by the fresh concentrated *Sphingobium* sp. cells grown on limonene as sole carbon source (Figure 26) or on glucose (Figure 27). In both cases the maximal *R*-(+)-α-terpineol concentration (10-12 $g.L^{-1}$ organic phase) was obtained after 72 h reaction with a yield of ~ 75% and a production rate of 0.25 $g.L^{-1}.h^{-1}$. These results demonstrate that, just like with *Fusarium oxysporum* (MARÓSTICA JR.; PASTORE 2007b) and in contrast with *Penicillium digitatum* (TAN; DAY; CADWALLADER, 1998), the enzyme was not inducible. The only need for the

culture medium was to support biomass growth, suggesting that, following a growing trend (PANDEY *et al*., 2000a,b,c), agricultural by-products such as cassava wastewater (MARÓSTICA JR.; PASTORE 2007) might be used as culture medium for the biocatalyst production. This behaviour was markedly different from, for example, that observed for the highly inducible α-pinene oxide lyase of *Pseudomonas rhodesiae* (FONTANILLE; LARROCHE 2003).

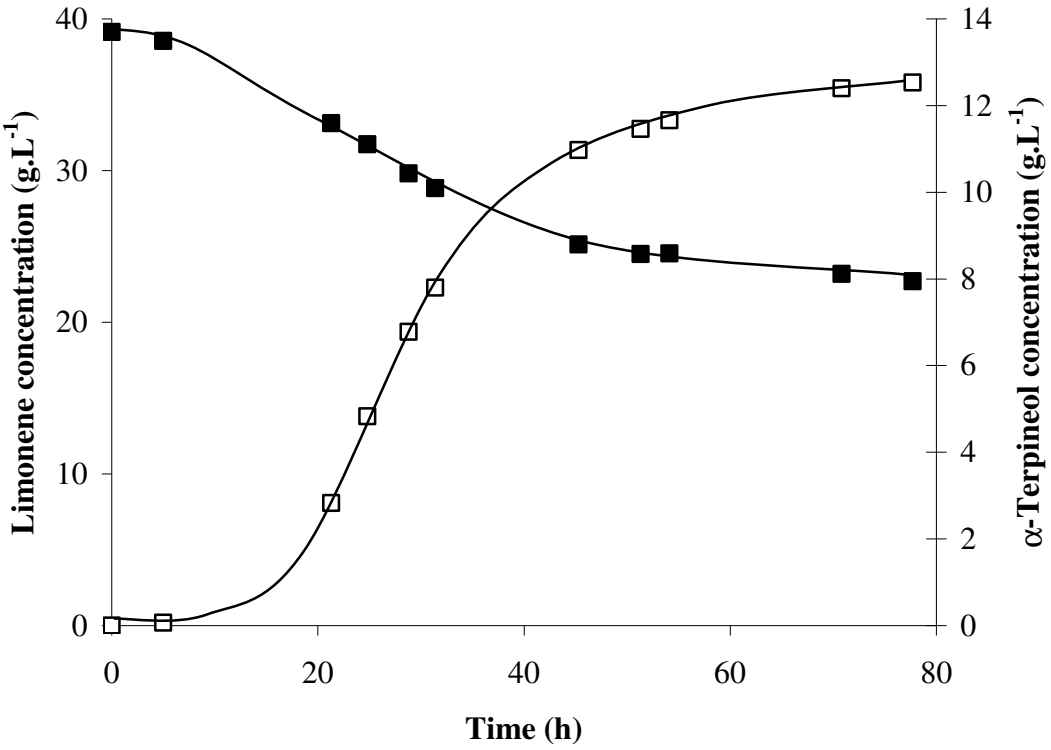


Figure 27. Bioconversion of *R*-(+)-limonene (■) to *R*-(+)-α-terpineol (□) by fresh *Sphingobium* sp. recovered from the pre-culture grown on glucose as sole carbon source (non induced biomass). *n*-Hexadecane was used as organic phase.

3.4. *n*-Hexadecane versus sunflower oil

In this study, the substitution of the traditional *n*-hexadecane (BICAS *et al*., 2008b) as organic phase for bioconversion on biphasic systems was tested. Considering the characteristics of the candidates such as high hydrophobicity, low toxicity for the microorganism and environment, low volatility, low price and high availability, it was decided that vegetable oils were the most suitable option for this purpose. Moreover, for anaerobic bioconversions, like the one described in this paper (see item 3.5), vegetable oils might be an interesting choice over *n*-hexadecane since as the oxygen solubility is much higher in *n*-hexadecane than in vegetable oils (KUTTUVA; RESTREPO; JU, 2004; WANG, 2002) and the employment of *n*-hexadecane increases the oxygen transfer in the system (NIELSEN; DAUGULIS; MCLELLAN, 2003), acting as an oxygen vector (ROLS *et al*., 1990). Sunflower oil was chosen as standard vegetable oil due to its abundance and low cost in the local market. The problem, in this case, is that many microorganisms are able to grow on vegetable oils and this might impede the bioconversion process by a catabolic repression-type inhibition. However, *Sphingobium* sp. does not grow on sunflower oil. A little increase is even noticed in the first 24 h (OD_{600} from 0.3-0.55) but it remained stable till after 72 h. This slight rise in OD_{600} at the beginning was then proposed to be due the formation of a water-oil emulsion.

Surprisingly, the substitution of the standard organic phase by sunflower oil was not only possible but it also presented much better results. The α-terpineol production rate was approximately 4 times higher, the yield and maximal concentration were almost double and the time for maximal concentration was 30 h shorter when the organic phase consisted of sunflower oil (Figure 28). This better performance was not related to the nature of the vegetable oils, since similar results were also obtained for rapeseed oil (Figure 29). Moreover, better results were also noticed, although in a minor scale, in the well-established process for the conversion of α-pinene

oxide to isonovalal by permeabilized *P. rhodesia* cells (FONTANILLE; LARROCHE 2003). In this case, the substitution of *n*-hexadecane by sunflower oil increases the initial production rate and maximal concentration by ~ 50% (results not shown).

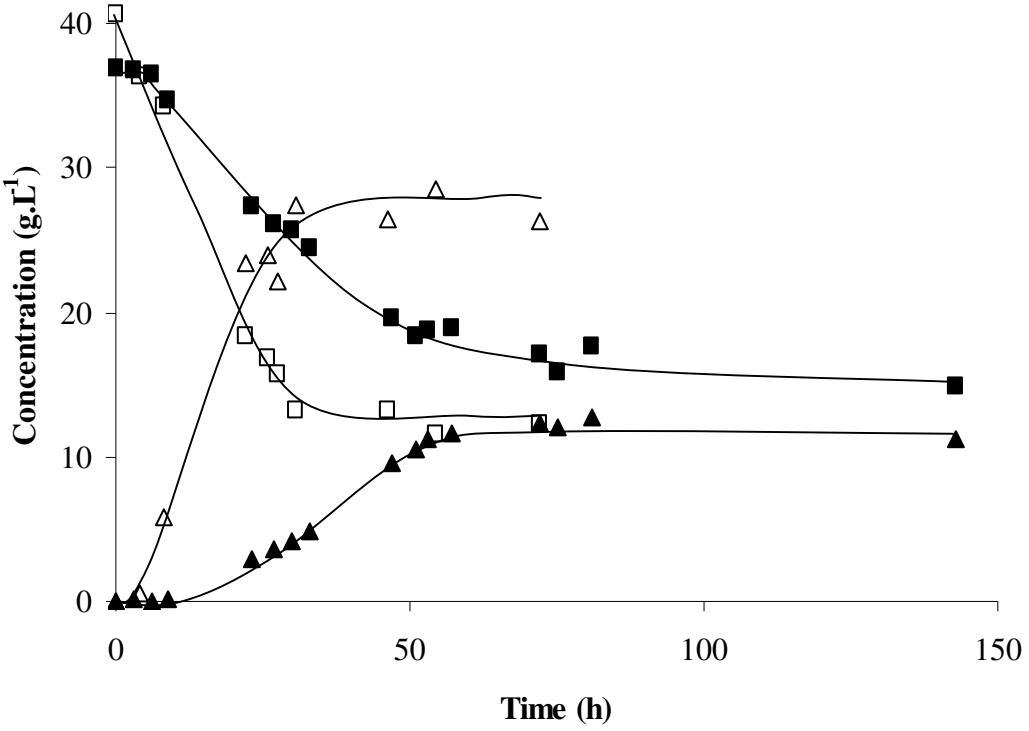


Figure 28. Bioconversion of *R*-(+)-limonene (squares) to *R*-(+)-α-terpineol (triangles) in *n*-hexadecane (black symbols) and sunflower oil (white symbols) by frozen *Sphingobium* sp. cells. Experiment carried out in 250 mL-conical flasks at 30°C and 200 rpm.

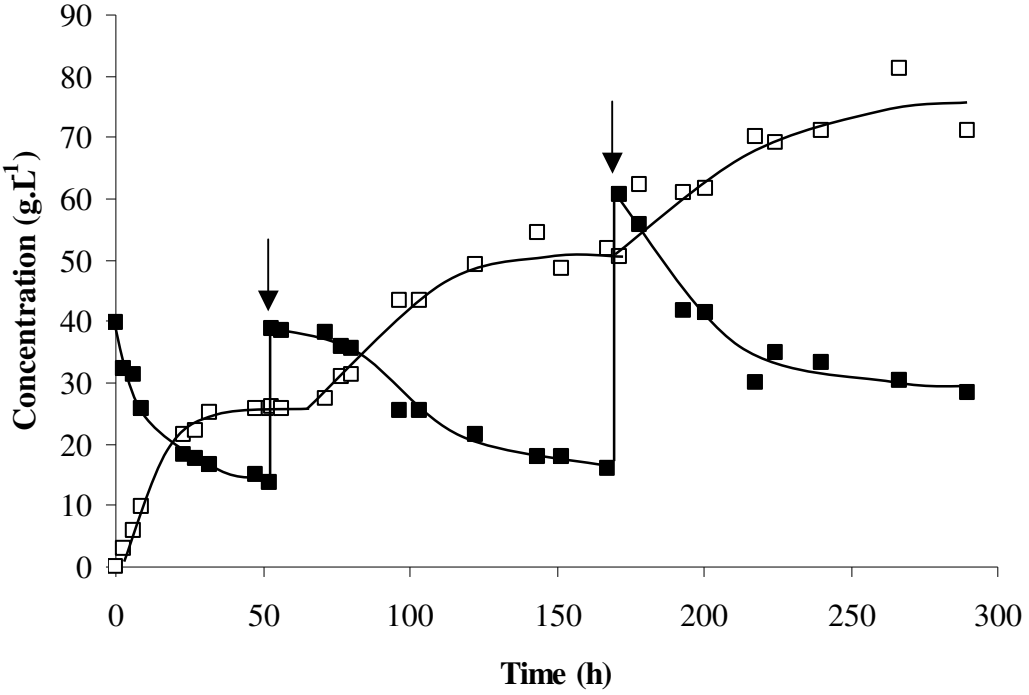


Figure 29. Bioconversion of *R*-(+)-limonene (■) to *R*-(+)-α-terpineol (□) in rapeseed oil by frozen *Sphingobium* sp. cells. Experiment carried out in 250 mL-conical flasks at 30°C and 200 rpm. The arrows represent the moment of substrate addition (500 μg).

When comparing the fresh (Figure 26) and frozen *Sphingobium* sp. biomass (Figure 28) for the bioconversion of *R*-(+)-limonene to *R*-(+)-α-terpineol in *n*-hexadecane as organic phase it is possible to notice a small decrease in the *R*-(+)-α-terpineol yield is evidenced for the frozen cells (56% against 73%), while the production rate and maximal *R*-(+)-α-terpineol concentration were equivalent in the two processes. This confirms that this biocatalyst might be stored at –18°C before being used in this process, resulting in no significant changes in the response.

3.5. Bioconversion in anaerobic conditions

The bioconversion in an anaerobic system showed that this reaction proceeded in the absence of O_2, suggesting that the enzyme was a cofactor-independent hydratase (CADWALLADER; BRADDOCK; PARISH, 1992) rather than a cytochrome P450-dependent monooxygenase system (TAN; DAY; CADWALLADER, 1998), the common catalyst of limonene in fungi (DUETZ *et al*. 2003). It might be seen in Figure 30 that the anaerobic conditions speeded up the production of *R*-(+)-α-terpineol in this bioconversion (from 0.26 to 1.1 $g.L^{-1}.h^{-1}$ and 1.0 to 2.5 $g.L^{-1}.h^{-1}$ when, respectively, *n*-hexadecane and sunflower oil were used) although the maximal concentrations of the product were always the same (~ 10 $g.L^{-1}$ for *n*-hexadecane and ~ 25 $g.L^{-1}$ for sunflower oil). For both organic phases the bioconversion yield was close to 100 %, what means a higher yield for *n*-hexadecane (formerly 56 %) and an equivalent yield for sunflower oil when comparing to the non-anaerobic conditions (Figure 28). Therefore, due to the differences in oxygen availability reported for the two organic phases tested, it is suggested that in non-anaerobic systems the oxidative metabolism is inhibited in sunflower oil and not in *n*-hexadecane, which explains the differences in the yields (Figure 28). Thus, it is believed that for sunflower oil only the transformation of limonene to α-terpineol takes place, while for *n*-hexadecane the two postulated pathways for limonene occurs simultaneously (BICAS *et al*., 2008b), decreasing the yield of α-terpineol.

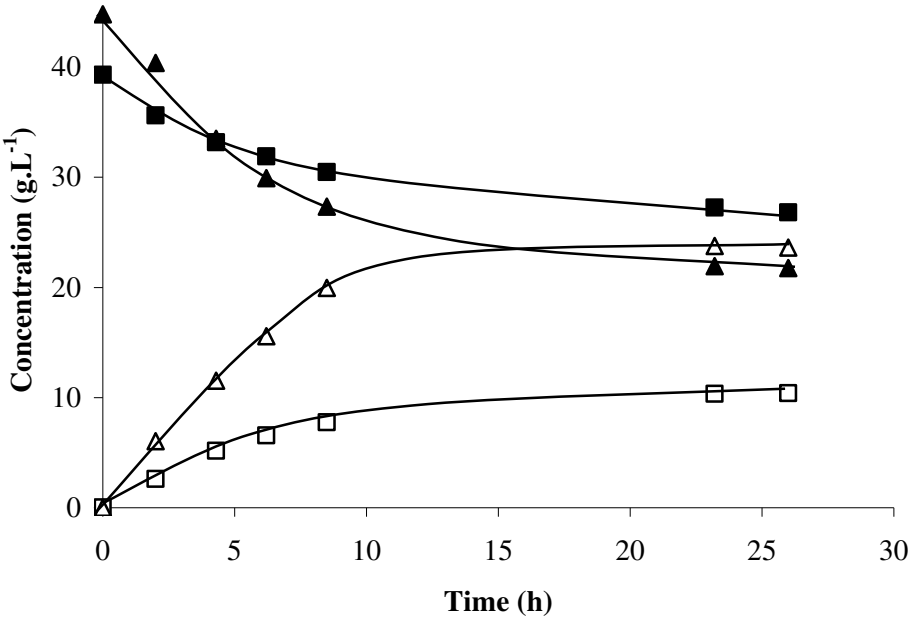


Figure 30. Anaerobic bioconversion of *R*-(+)-limonene (black symbols) to *R*-(+)-α-terpineol (white symbols) in *n*-hexadecane (squares) and sunflower oil (triangles) by frozen *Sphingobium* sp. biomass. Experiment carried out in 250mL conical flask with rubber stoppers at 30°C and 200 rpm. N_2 was flushed (c.a. 0.5 $L.min^{-1}$) for 5 min at the beginning and after each sampling.

3.6. Biotransformation in bioreactor

It is shown in Figure 31 that the bioconversion of *R*-(+)-limonene to *R*-(+)-α-terpineol is feasible in bioreactors, indicating that a scale-up of this process yielding comparable results is possible. Moreover, the bioconversion profile is analogous to that obtained in common conical flasks, with the same maximal concentration (~ 25 $g.L^{-1}$) and yields (~ 100%), although the production rate, as expected, was higher (2.0 $g.L^{-1}.h^{-1}$ against 1.0 $g.L^{-1}.h^{-1}$ in conical flasks) due to the high agitation and to the consequent increase in the mass transfer efficiency.

The end of α-terpineol production was also an object of study in this paper. Curiously, it was possible to observe that biotransformations always stopped when substrate concentration reached c.a. 20 $g.L^{-1}$. This phenomenon might be due to two main reasons: (i) as already stated, limonene is a toxic compound to bacteria and maybe the detoxification process stopped when the substrate concentration reached a critical concentration (in this case 20 $g.L^{-1}$). (ii) Or, the low accessibility of the substrate to the enzyme at low limonene concentrations (in this case <20 $g.L^{-1}$). In both cases, the addition of substrate would restart the α-terpineol production. Another cause may be the loss of enzymatic activity over time. This is at least partially true as seen by the lower reaction rates after the substrate addition (Figures 29 and 31) and the lower enzymatic activity of a biomass pre-treated at 30°C and 200 rpm for 40h (Figure 32). In the last case, the bioconversion proceeded under a lower yield (45% against 56%) and with the half production rate and maximal product's concentration (0.13 $g.L^{-1}.h^{-1}$ and 5.5 $g.L^{-1}$, respectively) when compared to the conventional process (Figure 28). Still, the exact mechanism of this phenomenon is yet unclear.

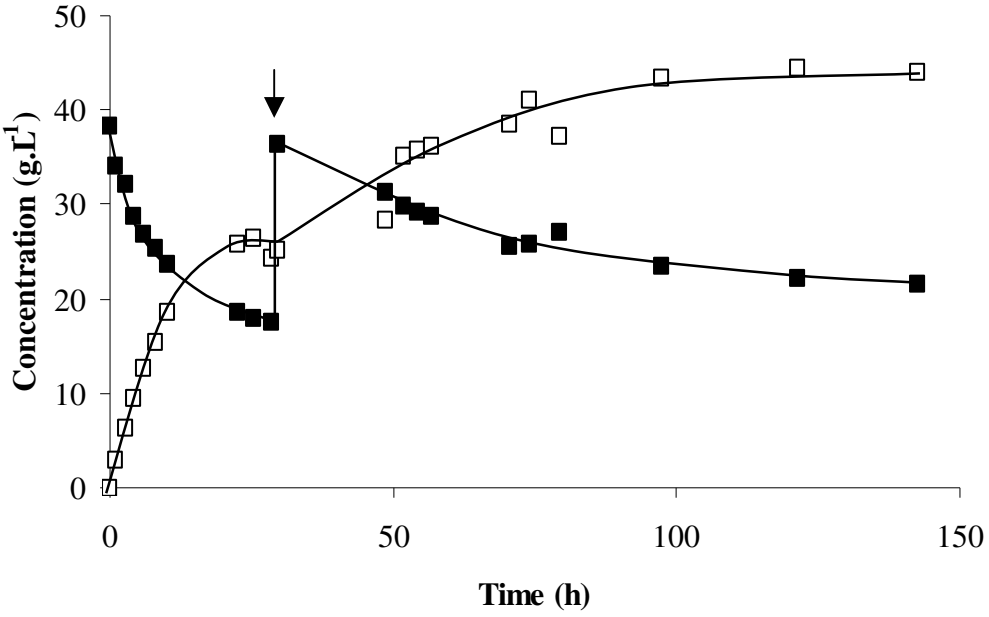


Figure 31. Bioconversion of *R*-(+)-limonene (■) to *R*-(+)-α-terpineol (□) by frozen *Sphingobium* sp. biomass in a 500mL bioreactor operated at 30°C/800 rpm and no aeration. Sunflower oil was used as organic phase. The arrow represents the moment of substrate addition (20 $g.L^{-1}$).

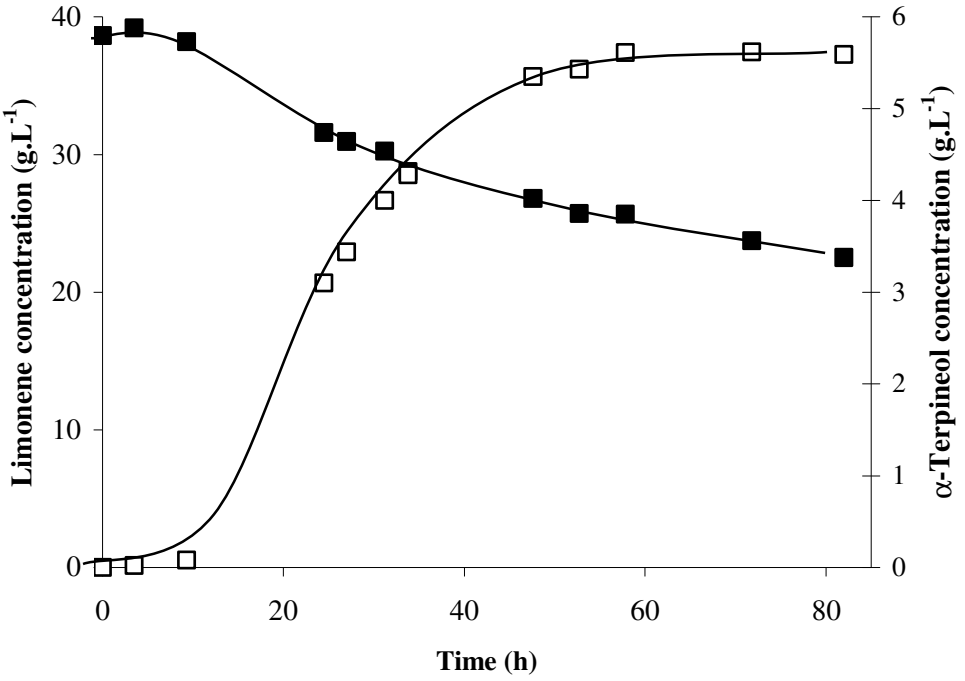


Figure 32. Bioconversion of *R*-(+)-limonene (■) to *R*-(+)-α-terpineol (□) by a frozen *Sphingobium* sp. biomass treated for 40 h at 30°C and 200 rpm in the presence of the same volume of *n*-hexadecane. The substrate was added to the organic phase at 0 h.

Amazingly, the strain tested in this study is very resistant to limonene. It was evidenced that α-terpineol was still produced when the bioconversion was carried out in a biphasic medium whose organic phase consisted of pure limonene (25 mL in 25 mL of aqueous phase). The production rate, which might be considered as the maximal rate (substrate saturation), was estimated as 4 $g.L^{-1}.h^{-1}$ and the maximal concentration was close to 55 $g.L^{-1}$ organic phase, achieved after 72-96 h. This is not the highest α-terpineol concentration possible from this process, since 120 to 130 $g.L^{-1}$ organic phase of α-terpineol was obtained when using an initial substrate concentration of 160 $g.L^{-1}$ in sunflower oil (data not shown).

3.7. Recovery of the product

The sunflower oil from the end of the bioconversion shown in Figure 28 was recovered for the isolation of the produced α-terpineol. For this purpose, 12 mL of oil was extracted with the same volume of 96% ethanol and 10 mL of the resulting alcoholic layer was evaporated in a vacuum roto-evaporator operated at 45-50 °C. The 258 mg of the yellow oily liquid obtained consisted of 85% (GC-FID area) *R*-(+)-α-terpineol (ee ~ 100%), 3% (GC-FID area) limonene, among others, equivalent to 219 mg of pure *R*-(+)-α-terpineol or ~ 22 g per liter of sunflower oil. The overall recovery yield was close to 85%.

4. Conclusion

In this chapter, a method for converting *R*-(+)-limonene into high concentrations of *R*-(+)-α-terpineol was presented. This is an anaerobic biotransformation, catalyzed by a non-inducible, cofactor independent and enantiospecific hydratase, capable of converting *S*-(–)-limonene to *S*-(–)-α-terpineol as well. It was demonstrated that the use of unconventional organic phases in biphasic medium processes is not only possible but also a very profitable alternative. This technique might be tested in other bioprocesses in which a significant reduction in the cost of production is required. The use of vegetable oils as organic phase is specially indicated for anaerobic bioconversions, since it inhibits the oxidative metabolism by creating an oxygen barrier stronger than those found in hydrocarbons.

The concentration of *R*-(+)-α-terpineol obtained (~ 25 g per liter of sunflower oil), which can be enhanced by the sequence substrate addition of increasing the initial substrate concentration, is by far the highest ever described for the bioproduction of this alcohol. The biotransformation in bioreactor increases the production rate with no alterations in yield and

maximal product concentration. The α-terpineol present in the vegetable oil is easily recovered and partially purified by a simple ethanolic extraction followed by a vacuum evaporation. Studies are already underway to optimize the bioconversion conditions and to improve the production of α-terpineol.

CAPÍTULO 7

α-TERPINEOL: UM BIOAROMA BIOATIVO

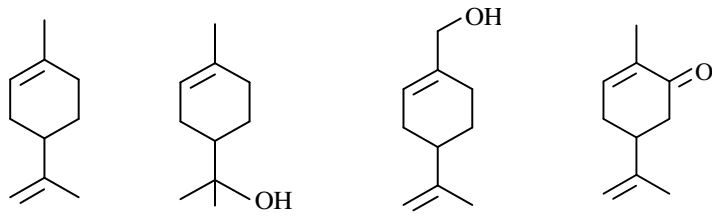
1. Introdução

A ação antioxidante de um composto confere a ele a capacidade de atuar como preservante em alimentos, fármacos e cosméticos, prevenindo a oxidação e degradação de seus componentes. Em geral, para este fim, tem-se empregado substâncias sintéticas, como o BHT, embora a tendência seja de substituí-las por compostos naturais como, por exemplo, os óleos essenciais, ricos em terpenos e terpenóides. Assim, presume-se que monoterpenos e monoterpenóides isolados também possam ser empregados com o mesmo objetivo.

Diversas técnicas têm sido desenvolvidas para a avaliação da atividade antioxidante contra espécies reativas de oxigênio (ROS), o que é principalmente justificado pela participação das ROS no desenvolvimento de diversas doenças, em especial o câncer, doenças cardiovasculares, desordens inflamatórias e neurodegenerativas e nos processos de envelhecimento (DÁVALOS; GÓMEZ-CORDOVÉS; BARTOLOMÉ, 2004; LAGUERRE; LACOMTE; VILLENEUVE, 2007; VALKO *et al*., 2004). Um desses métodos consiste na determinação do potencial de seqüestro do radical livre 2,2-difenil-1-picril-hidrasil (DPPH), cuja tonalidade azul-roxo (absorbância máxima ~515 ηm) é gradativamente desbotada ao ganhar um elétron. É, portanto, um método indireto simples e acurado de determinação de atividade antioxidante, largamente empregado para sucos ou extratos de frutas e vegetais e baseado na

transferência de elétrons. Contrariamente, o ensaio de capacidade de absorção de radical de oxigênio (ORAC) é um método direto baseado em transferência de átomos de hidrogênio, que mede a habilidade de compostos hidrofílicos ou lipofílicos no combate a reações de oxidação em cadeia com radicais peroxil. Apesar da diversidade de técnicas, o maior problema encontrado é a falta de ensaios validados que podem medir de forma confiável a capacidade antioxidante de alimentos e amostras biológicas (HUANG; OU; PRIOR, 2005). Assim, as melhores técnicas para avaliar a proteção de um composto contra o estresse oxidativo ainda são os estudos biológicos *in vitro* (culturas celulares) e *in vivo*.

Os processos citados em grande parte dessa tese resultam basicamente em um único produto: o α-terpineol (Figura 33). Como citado anteriormente, este composto de aroma é uma das substâncias mais frequentemente usadas na indústria de aromas, sendo encontrado especialmente na formulação de cosméticos e produtos de limpeza (BAUER; GARBE. SURBURG, 2001; SINGH *et al*. 1998). Porém, ele é relativamente abundante e com valor comercial bastante baixo, o que de certa forma tornam desinteressantes os processos biotecnológicos para sua obtenção. Contudo, a descoberta de novas funcionalidades e aplicações para esse bioaroma poderia torná-lo mais atrativo e valorizado, como acontece com alguns de seus análogos monoterpênicos.



Limoneno α-Terpineol Álcool perílico Carvona

Figura 33. Limoneno e seus derivados oxigenados citados nesse estudo.

O álcool perílico (Figura 33), por exemplo, é um dos monoterpenóides que mais se destacam. Além de atuar como agente de aroma, com emprego diversificado, esse derivado do limoneno apresenta ainda aplicação potencial na indústria farmacêutica já que diversos estudos *in vitro* e apontam para sua capacidade de inibir a proliferação de linhagens de células cancerosas de pulmão (YERUVA *et al*., 2007; XU *et al*., 2004), mama (YURI *et al*., 2004), cólon (BARDON *et al*., 2002) e próstata (CHUNG *et al*., 2006), muitas vezes induzindo a apoptose. Esse álcool também demonstrou capacidade de prevenir o desenvolvimento de diversos tipos de cânceres, como cólon, pele, pulmão, próstata, fígado, mama e pâncreas, em animais de experimentação (BELANGER, 1998; CROWELL, 1999). Encorajados por tais relatos, estudos clínicos ainda comprovaram seu potencial na regressão de gliomas malignos do sistema nervoso central, mostrando que o álcool perílico é um composto bastante promissor para o tratamento de tumores cerebrais, especialmente nos casos de insucesso com os métodos convencionais (DA FONSECA *et al*., 2006a,b; DA FONSECA *et al*., 2008). Outro exemplo é a carvona (Figura 33), cujo aroma característico (de menta para o isômero *R*-(–) ou de cominho e endro para o isômero *S*-(+)) encontra emprego diversificado na indústria de alimentos, bebidas e de higiene bucal (BAUER; GARBE. SURBURG, 2001), além de poder ser empregada na inibição de brotamento em batatas, como agente antimicrobiano ou repelente de insetos, entre outros. Essa cetona ainda é reconhecida como um agente eficaz no aumento da expressão da enzima detoxificante glutationa S-transferase, o que resulta em uma ação quimiopreventiva, uma vez que a atividade anticarcinogênica é correlacionada com a habilidade de induzir a expressão de enzimas detoxificantes (DE CARVALHO; DA FONSECA, 2006b). Apesar disso, quando comparada ao álcool perílico, a carvona apresenta menor expressão no combate de doenças.

Nesse Capítulo o α-terpineol será avaliado quanto a sua atividade antioxidante (ensaio de DPPH e ORAC) e ação antiploriferativa contra nove linhagens de células tumorais, sendo os resultados comparados a alguns de seus análogos terpênicos a fim de propor novas aplicações para este álcool.

2. Material e Métodos

2.1. Reagentes

Os padrões de α-terpineol (Aldrich, 90% pureza), (+)-α-terpineol (Fluka, ≥ 97% pureza), *R*-(–)-carvona (Merck, > 99% pureza), *S*-(–)-álcool perílico (Aldrich, 96% pureza), *R*-(+)-limoneno (Fluka, ≥ 98% pureza), radical livre 2,2-difenil-1-picril-hidrasil (DPPH) (Sigma), ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico (Trolox) (Aldrich, 97% pureza), fluoresceína sódica (Vetec) e 2,2'-azo-bis-(2-metil-amidinopropano)-di-hidrocloreto (AAPH) (Aldrich, 97% pureza) foram adquiridos com o fornecedor autorizado.

2.2. Determinação do potencial antioxidante *in vitro*

2.2.1. Ensaio de seqüestro do radical DPPH

A determinação do potencial antioxidante pelo método do radical DPPH foi uma adaptação do método sugerido por El-Ghorab, El-Massry, e Shibamoto (2007). Inicialmente foram preparadas soluções alcoólicas de diferentes concentrações dos terpenos a serem analisados. Posteriormente, 1 mL de cada solução foi adicionada ao mesmo volume de uma solução 40 $mg.L^{-1}$ de DPPH em metanol, sendo vigorosamente agitada e mantida no escuro por 30 min. Na seqüência o decréscimo na absorbância de cada amostra foi avaliada a 517 ηm contra etanol puro (branco). Os resultados, baseados no percentual de redução (ou inibição) do radical

DPPH [($Abs_{t=0}$ – $Abs_{t=30min}$)/$Abs_{t=0}$], foram obtidos de três replicatas independentes e comparados ao índice de atividade antioxidante proposto por Scherer e Godoy (2009). O índice IC_{50}, concentração que resulta em uma redução (ou inibição) de DPPH, foi determinado por regressão linear.

2.2.2. Método de capacidade de absorção de radical de oxigênio (ORAC)

O método de ORAC se baseia na ação protetora de um composto contra degradação da fluoresceína por radicais peroxil gerados pela degradação térmica do AAPH e é avaliada pelo decaimento da fluorescência do meio reacional ao longo do tempo, que segue uma cinética de ordem zero (DÁVALOS; GÓMEZ-CORDOVÉS; BARTOLOMÉ, 2004). Os resultados, expressos como μmol de Trolox (antioxidante padrão) equivalente por μmol de amostra, são baseados na Área Sob a Curva de decaimento da fluoresceína ao longo do tempo (ASC) e na Área líquida (AL), calculadas de forma aproximada conforme as Equações 1 e 2:

$$ASC \cong 1 + \sum_{i=1}^{80} \frac{f_i}{f_0} \qquad \text{Eq. 1,}$$

Onde f_0 é a fluorescência inicial (t = 0) e f_i é fluorescência lida a t = i (minutos).

$$AL = ASC_{amostra} - ASC_{branco} \qquad \text{Eq. 2}$$

Para esse experimento, uma adaptação do método descrito por Dávalos, Gómez-Cordovés e Bartolomé (2003), a reação foi realizada em tampão fosfato 75 mM a pH 7,4 em um volume final de 200 μL. As amostras a serem testadas e o padrão Trolox foram diluídas em etanol a diferentes concentrações (carvona: de 5 a 1.000 $mg.L^{-1}$; álcool perílico de 5 a 1.000 $mg.L^{-1}$; α-terpineol: de 0,1 a 20 $mg.L^{-1}$; Trolox: de 25 a 800 μM). Assim, 20 μL de cada diluição e 120 μL de fluoresceína (concentração final de 70 ηM) foram misturados em um dos 96 poços de uma

microplaca. Posteriormente, 60 μL de uma solução de AAPH (concentração final de 12 mM) foi adicionada rapidamente, sendo a microplaca imediatamente transferida para o leitor fluorimétrico de microplacas (NOVOstar®, BMG Labtech – Offenburg, Alemanha), mantido a 37 °C. Foram efetuados 80 ciclos de leitura, em um total de 80 min. Um experimento branco, sem antioxidante (20μL de tampão fosfato + 120 μL de fluoresceína + 60 μL de AAPH) foi considerado para o cálculo de AL. As curvas de AL *versus* Concentração foram traçadas para cada amostra e comparadas à curva padrão de Trolox. A equivalência em Trolox foi então dada pelo coeficiente angular da curva Concentração de Trolox (μM) *versus* Concentração de amostra (μM). Todos os ensaios foram efetuados em três replicatas independentes.

2.3. Atividade antiploriferativa *in vitro*

2.3.1. Linhagens celulares e cultivo

Foram empregadas sete linhagens celulares de diferentes origens histológicas e embrionárias humanas, todas cedidas pelo *National Cancer Institute* (NCI – EUA). São elas: K-562 (leucemia mielóide crônica), MCF-7 (adenocarcinoma de mama), NCI-H460 (adenocarcinoma de pulmão, células tipo não-pequenas), PC-3 (adenocarcinoma de próstata), HT-29 (adenocarcinoma de cólon), 786-O (adenocarcinoma de rim) e NCI-ADR/RES (adenocarcinoma de mama, com fenótipo resistência multidroga).

As culturas estoques foram conservadas em meio de cultura RPMI-1640 (Gibco) com 5% de soro fetal bovino (SFB) (Gibco) sem antibiótico, sendo esse adicionado (gentamicina, 50 $\mu g.mL^{-1}$) apenas nas células submetidas ao ensaio antiproliferativo. As células foram mantidas em frascos de $25cm^2$, com 5mL de meio RPMI/SFB e repicadas semanalmente. Cada frasco foi incubado a 37ºC em atmosfera de 5% de CO_2 e ambiente úmido.

Para as células aderidas, cujo crescimento ocorre em monocamada, a suspensão celular foi preparada esgotando-se o meio de cultura e lavando-se por 10 vezes consecutivas com tampão de Hank (Sigma). Após o esgotamento do tampão, foram adicionados 500 μL de tripsina e logo em seguida o meio RPMI/SFB. No caso das células não aderidas (K-562), a suspensão celular foi preparada transferindo-se um volume previamente determinado para uma nova placa de 25 cm^2, sendo o volume final ajustado para 5 mL.

2.3.2. Determinação da atividade antiproliferativa

Para a avaliação da atividade anticâncer 100 μL de suspensão celular em RPMI/SBFB/gentamicina, nas suas respectivas densidades de inoculação (Tabela 14), foram transferidas para placas de 96 poços e incubadas a 37ºC por 24h em atmosfera de 5% de CO_2 e ambiente úmido.

Tabela 14. Densidade de inoculação das linhagens celulares nos ensaios anticâncer.

Linhagem celular	Densidade de inoculação ($x\ 10^4$ células.mL^{-1})
NCI-H460	4
MCF-7	6
NCI-ADR/RES	5
HT-29	4
PC-3	5
786-O	4,5
K-562	4

A solução estoque de amostra (0,1 $g.mL^{-1}$ em DMSO) foi diluída em diferentes concentrações (0,25; 2,5; 25 e 250 $mg.L^{-1}$, em RPMI) e transferidas (100 μL) para os respectivos poços da placa (T). Um grupo controle não teve adição de amostra (C) e outro grupo, também sem adição de amostra, foi lido em tempo zero (T_0). A placa foi então incubada a 37ºC por 48h em atmosfera de 5% de CO_2 e ambiente úmido. Posteriormente, 50 μL de uma solução de ácido tricloroacético 50% foi adicionada e a placa foi mantida a 4°C por 30 min, a fim de fixar as células. Após lavagem e secagem, determinou-se o número de células por quantificação espectrofotométrica (540 ηm) do conteúdo protéico celular total, utilizando 50 μL por poço de sulforrodamicina B (0,4 $g.mL^{-1}$, em ácido acético 1%, por 30 min a 4°C) como indicador da quantidade de células viáveis (SKEHAN; SCUDEIRO; 1990). Doxorrubicina foi empregada como controle positivo. A partir dos gráficos de % Inibição ou morte celular *versus* Concentração de amostra serão obtidos os valores de TGI (*Total Growth Inhibition* ou Inibição Total do Crescimento) calculados a partir da Equação 3:

$$100\ x\ (T - T_0) / (C - T_0) = 0 \qquad \text{Eq. 3}$$

Onde T_0 e T correspondem, respectivamente, aos valores de absorbância no momento que as células receberam a amostra (tempo zero) e após o tratamento; C indica os valores de absorbância das células não tratadas (controle) após o mesmo período de tratamento.

3. Resultados

3.1. Atividade antioxidante pelo método de seqüestro do radical DPPH

A atividade antioxidante dos monoterpenóides carvona, álcool perílico e α-terpineol revelou que, em elevadas concentrações, todos eles possuem a capacidade de seqüestrar radicais livres. A carvona (IC_{50} = 32,1 $g.L^{-1}$) mostrou-se a mais potente dos três, apresentando um valor de IC_{50} dez vezes superior ao do α-terpineol (IC_{50} = 332,8 $g.L^{-1}$) e 23 vezes superior ao do álcool perílico (IC_{50} = 738,3 $g.L^{-1}$) (Figura 34). O melhor desempenho da carvona nesse ensaio é bastante compreensível visto que sua estrutura, com uma dupla ligação C=C conjugada a uma carbonila (Figura 33), possui uma maior capacidade de captura de radicais livres quando comparada aos outros terpenóides testados. No entanto, todos eles apresentam uma capacidade baixíssima de seqüestro de radicais livres quando comparada a antioxidantes convencionais. Para se ter um exemplo, Scherer e Godoy (2009) determinaram o índice de atividade antioxidante (IAA) para o ácido gálico, BHA e ácido ferrúlico, de 27, 9 e 5,3, respectivamente, enquanto que os valores de IAA para a carvona, álcool perílico e α-terpineol encontrados nesse estudo foram de 0,001, 5.10^{-5} e 0,0001. Para os autores, valores de IAA abaixo de 0,5 seriam considerados com baixa atividade antioxidante.

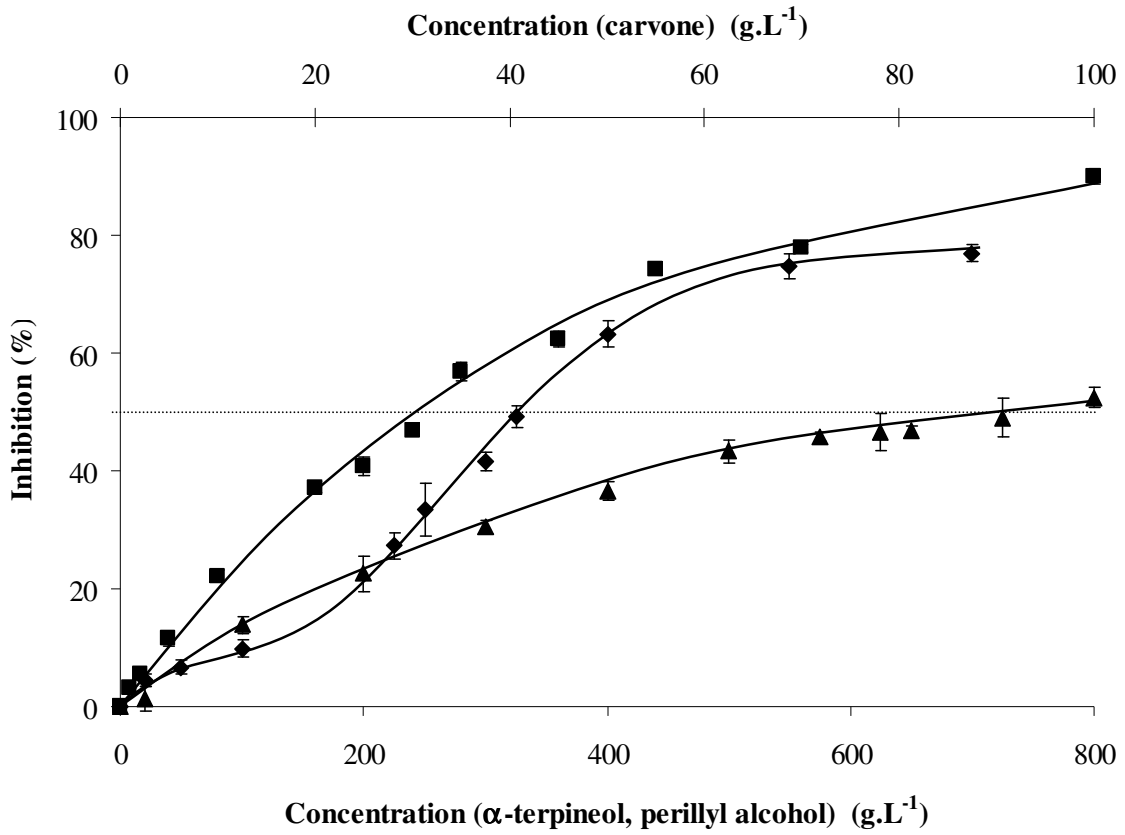


Figura 34. Inibição do radical DPPH por soluções alcoólicas dos monoterpenóides carvona (■), álcool perílico (▲) e α-terpineol (◆). A linha tracejada representa a redução de 50 % do DPPH.

3.2. Atividade antioxidante pela metodologia de ORAC

A Figura 35 apresenta os valores de ORAC obtidos para cada um dos monoterpenóides analisados. Curiosamente, a carvona, que no ensaio de DPPH demonstrou a maior atividade antioxidante, foi o composto com menor atividade nesse teste (0,12 µmol de Trolox equiv./µmol de carvona), seguido pelo álcool perílico (0,68 µmol de Trolox equiv./µmol álcool perílico). Já o α-terpineol apresentou a maior atividade antioxidante (2,72 µmol de Trolox equiv./µmol α-terpineol), com um valor de ORAC semelhante ao do antioxidante sintético BHA (2,43 µmol de Trolox equiv./µmol BHA) encontrado por Dávalos, Gómez-Cordovés e Bartolomé (2003). O

ensaio com outro padrão de α-terpineol ((+)-α-terpineol) apresentou praticamente o mesmo valor (2,75 µmol de Trolox equiv./µmol α-terpineol).

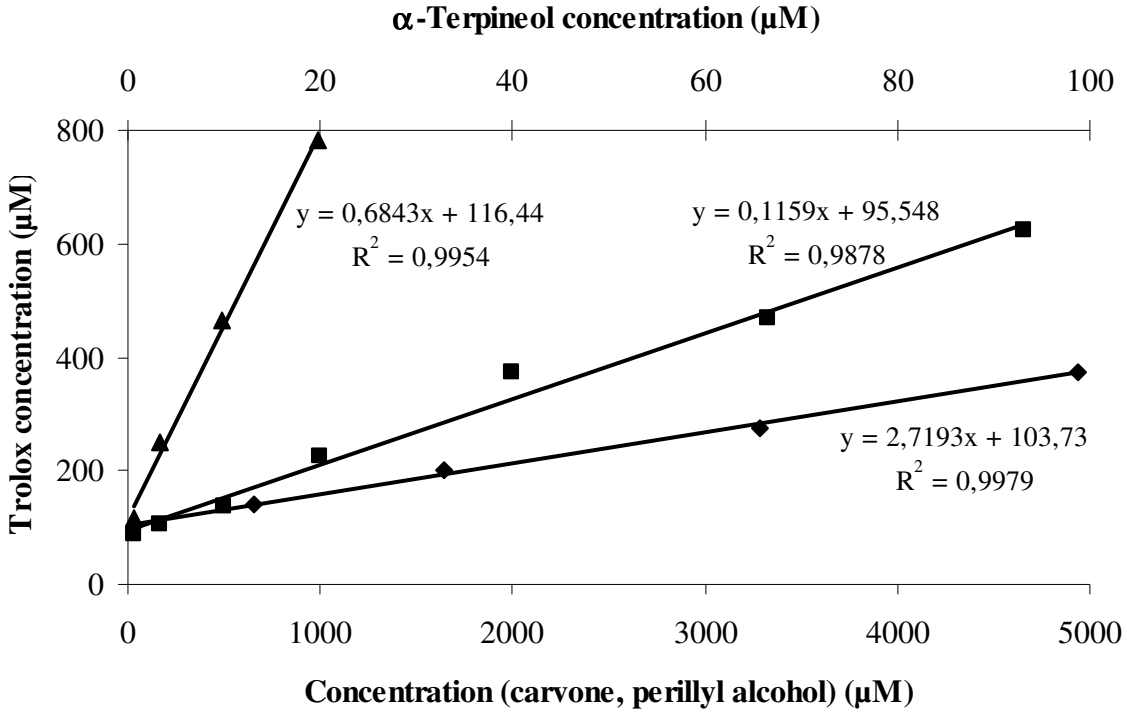


Figura 35. Equivalência de Áreas liquidas (AL) no ensaio de ORAC: relação entre a concentração de Trolox (µM) e a concentração de carvona (■), álcool perílico (▲) e α-terpineol (◆). Os coeficientes angulares das retas representam o valor de ORAC (µmol de Trolox por µmol de amostra).

3.3. Atividade antiproliferativa *in vitro*

As Figuras 36, 37 e 38 apresentam o porcentual de crescimento das células para as amostras testadas. A linha de 0% foi traçada para a visualização do TGI (*Total Growth Inhibition* ou Inibição Total do Crescimento), que é a concentração necessária para que ocorra o efeito citostático (sem crescimento celular). Esses valores podem ser visualizados na Tabela 15. Os números negativos representam o percentual de morte celular. Nota-se que o limoneno e o α-terpineol apresentam razoável atividade antiproliferativa, com TGI variando de 500 a 900 μM, para praticamente todas as linhagens testadas, à exceção das linhagens 786-O e HT-29, que não tiveram seu crescimento completamente inibido em nenhuma das concentrações testadas. De forma geral, o perfil de inibição de ambos monoterpenos testados são muito similares entre si e bem distantes do controle positivo doxorrubicina, cujos valores de TGI que chegam a ser de 10 a 600 vezes superior ao das amostras, dependendo da linhagem testada. Vale notar que a linhagem K-562 (leucemia mielóide crônica), mostrou-se consideravelmente mais sensível que as demais, especialmente na presença de α-terpineol. Já a linhagem HT-29 (adenocarcinoma de cólon) foi uma das mais resistentes, independentemente da amostra testada.

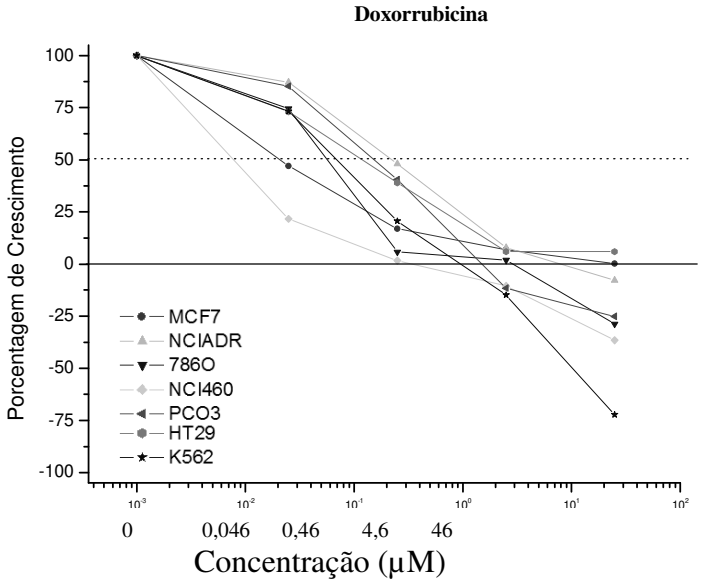


Figura 36. Atividade antiproliferativa da doxorrubicina para sete linhagens de células tumorais.

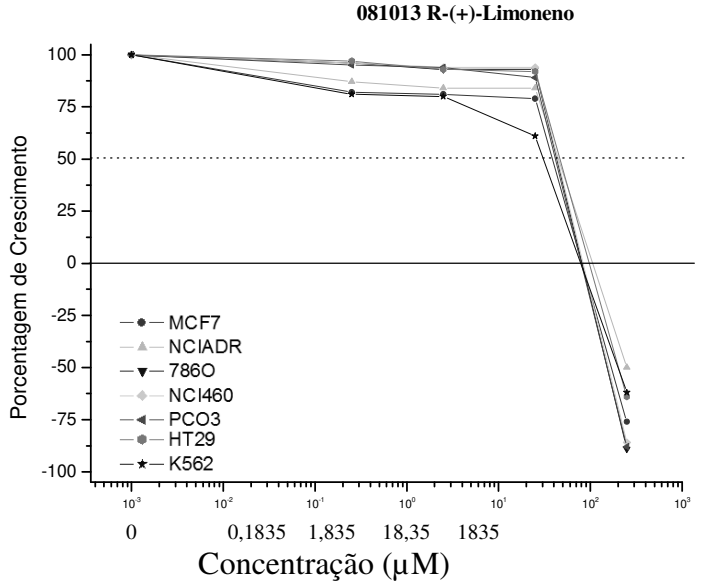


Figura 37. Atividade antiproliferativa do *R*-(+)-limoneno para sete linhagens de células tumorais.

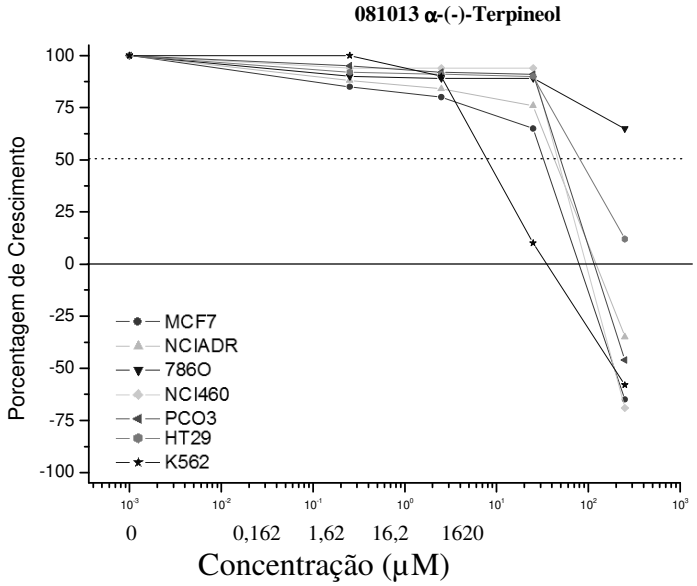


Figura 38. Atividade antiploriferativa do α-terpineol para sete linhagens de células tumorais.

Tabela 15. Valores de TGI[a] (μM) para a doxorrubicina, o *R*-(+)-limoneno e o α-terpineol em cada uma das linhagens testadas.

Amostra	Linhagem						
	MCF-7	NCI-ADR/RES	786-O	NCI-H460	PC-3	HT-29	K-562
Doxorrubicina	46	15,7	3,9	1,3	5,6	>46	1,7
R-(+)-limoneno	601	886	633	687	591	870	559
α-Terpineol	503	856	>1600	760	916	>1600	249

[a] TGI (*Total Growth Inhibition* ou Inibição Total do Crescimento) foi calculado a partir da equação: 100 *x* (T – T_0) / (C – T_0) = 0, onde T_0 e T correspondem, respectivamente, aos valores de absorbância no momento que as células receberam a amostra (tempo zero) e após o tratamento; C indica os valores de absorbância das células não tratadas (controle) após o mesmo período de tratamento.

4. Discussão

A procura por antioxidantes naturais para fins nutricionais, cosméticos e farmacológicos tem se tornado um dos principais desafios industriais e científicos dos últimos 20 anos (LAGUERRE, LACOMTE, VILLENEUVE, 2007). Eles podem atuar na preservação de alimentos ou ainda exercer efeitos biológicos pelo combate às espécies reativas de oxigênio (ROS).

Diversos efeitos biológicos têm sido atribuídos aos óleos essenciais, muitos dos quais caracterizados como antioxidantes (BAKKALI *et al*., 2007), que podem ser considerados naturais. A falta de estudos com padrões, no entanto, dificulta a determinação de sinergismo ou antagonismo que podem ocorrer nos óleos essenciais. Um dos poucos trabalhos com substâncias puras de óleos essenciais avaliou por dois modelos diferentes a atividade antioxidante de 13 monoterpenos (hidrocarbonetos), 34 monoterpenóides (oxigenados), 11 sesquiterpenos (hidrocarbonetos), 10 sesquiterpenóides (oxigenados), um diterpeno, sete derivados benzênicos e outros 22 componentes não terpênicos. A avaliação dos resultados indicou que, como esperado, os fenóis timol e carvacrol apresentaram os melhores resultados. Ademais, os monoterpenos terpinoleno, α- e γ-terpineno e os álcoois álcool perílico, nerol, *cis*-verbenol e geraniol apresentaram bons resultados. Já o α-terpineol e a carvona mostraram atividades relativamente baixas (RUBERTO; BARATTA, 2000). Com base nos resultados os autores sugeriram que grupos metilenos altamente ativados e que álcoois alílicos favorecem uma maior atividade antioxidante, o que pode ser questionável devido a algumas exceções a essa regra. De toda forma, observa-se a grande dificuldade em se relacionar estrutura química à atividade antioxidante.

Nesse estudo, os três compostos testados apresentaram atividade antioxidante bastante baixa para o ensaio de DPPH, com valores muito abaixo dos esperados para antioxidantes

convencionais (SHERER; GODOY, 2009), algo compreensível visto que óleos essenciais comumente apresentam fraco desempenho nesse ensaio (LOPES-LUTZ *et al*., 2008). Porém, a carvona mostrou os melhores resultados, provavelmente devido à sua dupla ligação conjugada à uma carbonila (Figura 33). No caso do ensaio de ORAC, essa não é uma metodologia comumente empregada para monoterpenóides, embora seja muito comum para extratos vegetais, plasma e fitoquímicos purificados (HUANG; OU; PRIOR, 2005). Interessantemente, os melhores resultados foram obtidos para o α-terpineol, seja o padrão Aldrich ou Fluka (poder rotacional positivo), com um valor de ORAC de 2,72-2,75 μmol de Trolox equivalente por μmol de α-terpineol

Uma variedade de terpenos tem sido relatada como agentes antitumorais, notadamente o limoneno, o álcool perílico, o mentol e a carvona. A atividade antitumoral desses compostos parece ser devida a diferentes mecanismos, seja pela indução das enzimas das fases I e II do metabolismo carcinogênico, resultando na detoxificação do carcinogeno, seja pela indução da apoptose ou pela inibição da isoprenilação pós-traducional das proteínas reguladoras do crescimento celular (CROWELL, 1999; WAGNER; ELMADFA, 2003). Os principais relatos de inibição da carcinogênese quimicamente induzida em roedores sugerem que o limoneno apresenta efeito protetor contra tumores de mama, pele, fígado, pulmão, pré-estômago, pâncreas e próstata (CROWELL, 1999; JIN *et al*., 2008) e, ao que se sabe, nenhuma publicação descreve o estudo de atividade antiproliferativa do α-terpineol. Nesse Capítulo foi demonstrado que o α-terpineol apresentou atividade antiproliferativa semelhante à do limoneno contra as linhagens MCF-7 (adenocarcinoma de mama), NCI-H460 (adenocarcinoma de pulmão, células tipo não-pequenas), PC-3 (adenocarcinoma de próstata), NCI-ADR/RES (adenocarcinoma de mama, com fenótipo resistência multidroga) e uma maior atividade para a linhagem K-562 (leucemia

mielóide crônica). Os valores de TGI (efeito citostático) são consideravelmente inferiores ao controle positivo doxorrubicina, mas são suficientes para encorajar estudos com animais para validar seu efeito antitumoral in vivo.

5. Conclusão

No futuro, aditivos alimentares deverão não só suprir as necessidades nutricionais e tecnológicas básicas. A tendência é que os ingredientes provenham de fontes naturais, tenham diferentes funções tecnológicas no alimento e ainda possam contribuir favoravelmente e de forma holística à promoção da saúde e bem estar do consumidor. São os já conhecidos alimentos funcionais, que devem se popularizar progressivamente.

Com esse estudo, o α-terpineol pode agora ser considerado um novo potencial aditivo funcional, uma vez que além de sua aplicação como agente de aroma ele também demonstrou capacidade antioxidante contra radicais peroxil, com ação quase três vezes mais intensa que o padrão Trolox nas concentrações e condições avaliadas. Esse álcool monoterpênico também apresentou ação antiproliferativa contra cinco linhagens tumorais humanas de quatro origens histológicas e embrionárias diferentes (leucemia, mama, pulmão, próstata), com um perfil bastante semelhante a seu precursor limoneno, já reconhecido por sua atividade anticarcinogênica *in vivo*. Futuramente, ensaios *in vivo* poderão confirmar seu potencial antitumoral e elevar ainda mais o interesse por esse composto. Nesse caso, fontes naturais de α-terpineol tornar-se-ão essenciais industrialmente.

CONCLUSÃO GERAL

Esta tese apresentou os resultados atingidos empregando processos biotecnológicos para a obtenção e otimização da produção de bioaromas, especialmente do monoterpenóide α-terpineol.

A literatura científica comprova o elevado interesse industrial por compostos de aroma naturais, especialmente aqueles obtidos por microrganismos. No entanto, a seleção de linhagens com elevada produção tem se mostrado essencial para o desenvolvimento de processos economicamente viáveis. Nesse estudo, a seleção de linhagens provenientes de fontes cítricas mostrou ser possível obter um grande número de microrganismos, especialmente bactérias Gram positivas, capazes de utilizar o *R*-(+)-limoneno como única fonte de carbono. No entanto, como aqui relatado, a acumulação de metabólitos de interesse nem sempre ocorre, o que pode ser devido à completa utilização do substrato para crescimento e produção de energia. Em contrapartida, o acúmulo de metabólitos de pode estar relacionado a uma "falha" no metabolismo (diferenças nas velocidades e atividades enzimáticas em uma via metabólica) ou a um metabolismo secundário que, no caso dos terpenos, compostos reconhecidamente tóxicos aos microrganismos, seria o metabolismo de xenobióticos. De toda maneira, torna-se muito importante conhecer as vias pelas quais os substratos são degradados para, aí então, delinear o melhor método para aumentar a recuperação do produto de interesse. No caso das bactérias *Pseudomonas rhodesiae* CIP 107491 e *P. fluorescens* NCIMB 11671, foram elucidadas as vias de degradação do α- e β-pinenos, ambas passando por intermediários aldeídicos e ácidos antes de integrarem a β-oxidação. Nesses casos, pode-se obter elevados rendimentos e produtividade de isonovalal partindo-se do precursor α-pineno óxido com ação de uma liase independente de

cofatores. Para *P. fluorescens*, outras duas rotas metabólicas para o limoneno foram relatadas, uma para crescimento e produção de energia, envolvendo intermediários alcoólicos, e outra responsável pela detoxificação, tendo como único produto (produto final) o α-terpineol. Uma via equivalente fora observada para o fungo produtor de lipase alcalina *Fusarium oxysporum* 152b, o que levou a um estudo do efeito das principais variáveis envolvidas nesse processo para posterior otimização das condições de produção e ao desenvolvimento de um processo integrado para a coprodução de lipase/α-terpineol. Foi demonstrado que o Planejamento Experimental, empregando matriz Placket-Burmann e um Delineamento Composto Central Rotacional, foi eficiente para a avaliação de 10 dos principais parâmetros. Em condições ótimas, a produção de α-terpineol pelo *F. oxysporum* atingiu 2,4 $g.L^{-1}$ após 72 h, uma concentração baixa se comparada aos quase 25 $g.L^{-1}$ atingidos pela bactéria *P. fluorescens* após 30 – 40 h em meio bifásico. Esses valores podem encorajar a aplicação da produção biotecnológica de α-terpineol. Todavia, esse produto não é suficientemente valorizado como seus equivalentes monoterpênicos como, por exemplo, o álcool perílico e a carvona, compostos com reconhecida propriedade anticarcinogênica inclusive em ensaios clínicos. Dessa forma, os estudos preliminares *in vitro* obtidos demonstraram o potencial funcional também para o α-terpineol, o que, se for confirmado *in vivo*, seguramente aumentará o interesse por tal composto.

REFERÊNCIAS BIBLIOGRÁFICAS

ABECITRUS – **Associação Brasileira dos Exportadores de Cítricos.** Subprodutos da Laranja. Disponível em <www.abecitrus.com.br/subprodutos_br.html>. Acessado em 03 dez. 2008.

ABRAHAM, W. R. – Phylogenetic Influences in Microbial Hydroxylation of Terpenoids. **World Journal of Microbiology and Biotechnology**. V. 10, n. 1, p. 88-92, 1994.

ABRAHAM, W. R.; HOFFMANN, H. M. R.; KIESLICH, K.; RENG, G.; STUMPF, B. – Microbial Transformations of Some Monoterpenoids and Sesquiterpenoids. In: **Enzymes in Organic Synthesis.** Ciba Foundation Symposium 111. London: Pitman. p. 146-160. 1985.

ADAMS, A.; DEMYTTENAERE, J. C. R.; DE KIMPE, N. - Biotransformation of (R)-(+)- and (S)-(–)-limonene to Alpha-terpineol by *Penicillium digitatum* - Investigation of the Culture Conditions. **Food Chemistry**. v. 80, n. 4, p. 525-534, 2003.

AGRAWAL, R.; DEEPIKA, N.; JOSEPH, R. – Strain Improvement of *Aspergillus* sp. and *Penicillium* sp. by Induced Mutation for Biotransformation of α-Pinene to Verbenol. **Biotechnology and Bioengineering.** V. 63, n. 2, p. 249-252, 1999.

AGRAWAL, R.; JOSEPH, R. – Bioconversion of Alpha Pinene to Verbenol by Resting Cells of *Aspergillus niger*. **Applied Microbiology and Biotechnology.** V. 53, p. 335-337, 2000a.

AGRAWAL, R.; JOSEPH, R. – Optimization of Conditions for the Biotransformation of Alpha Pinene to Verbenone by a *Penicillium* sp. **Journal of Food Science and Technology.** V. 37, n. 4, p. 430-432, 2000b.

ALEU, J.; COLLADO, I. G. – Biotransformations by *Botrytis* Species. **Journal of Molecular Catalysis B: Enzymatic.** V. 13, n. 4-6, p. 77-93, May 2001.

ALLAL, B. A.; EL FIRDOUSSI, L.; ALLAOUD, S.; KARIM, A.; CASTANET, Y.; MORTREUX, A. – Catalytic Oxidation of α-Pinene by Transition Metal Using t-Butyl Hydroperoxide and Hydrogen Peroxide. **Journal of Molecular Catalysis A: Chemical**. V. 200, n. 1-2, p. 177-184, 2003.

BAKKALI, F.; AVERBECK, S.; AVERBECK, D.; IDAOMAR, M. – Biological Effects of Essential Oils - A Review. **Food and Chemical Toxicology.** V. 46, n. 2, p. 446-475, Feb. 2007.

BALDERMANN, S.; NAIM, M.; FLEISCHMANN, P. – Enzymatic Carotenoid Degradation and Aroma Formation in Nectarines (*Prunus persica*). **Food Reserach International.** V.38, n. 8-9, p. 833-836, 2005.

BARDON, S.; FOUSSARD, V.; FOURNEL, S.; LOUBAT A. – Monoterpenes Inhibit Proliferation of Human Colon Cancer Cells by Modulating Cell Cycle-Related Protein Expression. **Cancer Letters.** V. 181, p. 187–194, 2002.

BARROS, F.F.C. – **Estudo das Variáveis de Processo e Ampliação de Escala na Produção de Biossurfactante por *Bacillus subtilis* em Manipueira.** Campinas: Unicamp, 2007 (Tese – Mestrado em Ciência de Alimentos).

BAUER, K.; GARBE, D.; SURBURG, H. – **Common Fragrance and Flavor Materials**: Preparation, Properties and Uses. 4th ed. Weinheim: Wiley - VCH, 2001. 293 p.

BELANGER, J.T. – Perillyl Alcohol: Applications in Oncology **Alternative Medicine Review.** V.3, n. 6, p. 448- 457, 1998.

BELL, S. G.; SOWDEN, R. J.; WONG, L. -L. Engineering the Haem Monooxygenase Cytochrome $P450_{cam}$ for Monoterpene Oxidation. **Chemical Communications.** V. 7, p. 635-636, 2001.

BERGER, R. G. – **Aroma Biotechnology.** Berlin: Springer-Verlag, 1995. 240 p.

BERGER, R.G.; DE BONT, J.A.M.; EGGINK, G.; DA FONSECA, M.M.; GEHREKE, M.; GROS, J.-B.; VAN KEULEN, F.; KRINGS, U.; LARROCHE, C.; LEAK, D. AND VAN DER WERF, M. – Biotransformations in the Flavour Industry. In: SWIFT, K.A.D. (Ed.). **Current Topics in Flavours and Fragrances.** Towards a New Millennium of Discovery. London: Kluwer Academic Publishers, 1999, pp. 139-170.

BERNHARDT, R. - Cytochromes P450 as Versatile Biocatalysts. **Journal of Biotechnology.** V. 124, n. 1, p. 128-145, 2006.

BEST, D. J.; FLOYD, N. C.; MAGALHAES, A.; BURFIELD, A.; RHODES, P. M. – Initial Enzymic Steps in the Degradation of Alpha-Pinene by *Pseudomonas fluorescens* NCIMB 11671. **Biocatalysis.** V. 1, n. 2, p. 147-159, 1987.

BHATTACHARYYA, P. K.; PREMA, B. R.; KULKARNI, B. D.; PRADHAN, S. K. – Microbiological Transformation of Terpenes: Hydroxylation of α-Pinene. **Nature.** V. 187, p.689-690, 1960.

BICAS, J. L.; BARROS, F. F. C.; WAGNER, R.; GODOY, H. T.; PASTORE, G. M. – Optimization of *R*-(+)-α-terpineol Production by the Biotransformation of *R*-(+)-Limonene. **Journal of Industrial Microbiology and Biotechnology**. V. 35, n. 9, p. 1061-1070, 2008a.

BICAS, J. L.; FONTANILLE, P.; PASTORE, G. M.; LARROCHE, C. – Characterization of Monoterpene Biotransformation in Two Pseudomonads. **Journal of Applied Microbiology**. V. 105, p. 1991, 2001, 2008b.

BICAS, J. L.; PASTORE, G. M. – Isolation and Screening of *d*-Limonene Resistant Microorganisms. **Brazilian Journal of Microbiology**. V. 38, p. 563-567, 2007.

BOGNOLO, G. – Biosurfactants as Emulsifying Agents for Hydrocarbons. **Colloids and Surfaces A: Physicochemical and Engineering Aspects.** V. 152, n. 1-2, p. 41-52, July 1999.

BOSSER, A.; BELIN, J. M. – Synthesis of β-Ionone in an Aldehyde/Xanthine Oxidase/β-Carotene System Involving Free Radical Formation. **Biotechnoogy Progress**. V. 10, n. 2, p. 129-133, 1994.

BRADDOCK, R. J.; CADWALLADER, K. R. – Bioconversion of Citrus *d*-Limonene. In: ROUSEFF, R. L.; LEAHY, M. M. (Eds.), **Fruit Flavors: Biogenesis, Characterization and Authentication**. Washington, DC: ACS Symp. Series N596, 1995, pp. 142-148.

BRADSHAW, W. H. – Cyclic Intermediates in the Bio-Oxidation of *d*-Camphor. **Federation Proceedings.** V. 18, n. 1, p. 196, 1959.

BRADSHAW, W. H.; CONRAD, H. E.; COREY, E. J.; GUNSALUS, I. C.; LEDNICER, D. – Microbiological Degradation of (+)-Camphor. **Journal of the American Chemical Society.** V. 81, n. 20, p. 5507, 1959.

CABRAL, J. M. S. – Biotransformations. In: RATLEDGE, C.; KRISTIANSEN, B. (Eds) **Basic Biotechnology**. 2nd ed. Cambridge: Cambridge University Press, 2001, Ch. 22, pp.471-501.

CADWALLADER, K. R.; BRADDOCK, R. J.; PARISH, M. E. – Isolation of α-Terpineol Dehydratase from *Pseudomonas gladioli*. **Journal of Food Science.** V. 57, n. 1, p. 241-244 and 248, 1992.

CADWALLADER, K.R.; BRADDOCK, R.J.; PARISH, M.E.; HIGGINS, D.P. – Bioconversion of (+)-Limonene by *Pseudomonas gladioli*. **Journal of Food Science.** V. 54, n. 5, p. 1241-1245, 1989.

CANTWELL, S. G.; LAU, E. P.; WATT, D. S.; FALL, R. R. – Biodegradation of Acyclic Isoprenoids by *Pseudomonas* Species. **Journal of Bacteriology.** V. 135, n. 2, p. 324-333, Aug. 1978.

CAOVILLA, M.; CAOVILLA, A.; PERGHER, S. B. C.; ESMELINDRO, M. C.; FERNANDES, C.; DARIVA, C.; BERNARDO-GUSMÃO, K.; OESTREICHER, E. G.; ANTUNES O. A. C. – Catalytic Oxidation of Limonene, α-Pinene and β-Pinene by the Complex [FeIII(BPMP)Cl(μ-O)FeIIICl$_3$] Biomimetic to MMO Enzyme. **Catalysis Today**. V. 133-135, p. 695. 2008.

CARRILLO, C.; TERUEL, J.A.; ARANDA, F.J.; ORTIZ, A. – Molecular Mechanism of Membrane Permeabilization by the Peptide Antibiotic Surfactin. **Biochimica et Biophysica Acta**. V. 1611, n. 1-2, p. 91-97, 2003.

ÇELIK, D.; BAYRAKTAR, E.; MEHMETOĞLU, Ü. – Biotransformation of 2-Phenylethanol to Phenylacetaldehyde in a Two-Phase Fed-Batch System. **Biochemical Engineering Journal.** V. 17, n. 1, p. 5-13, 2004.

CHANG, H. C.; GAGE, D. A.; ORIEL, P. J. – Cloning and Expression of a Limonene Degradation Pathway from *Bacillus stearothermophilus* in *Escherichia coli*. **Journal of Food Science.** v. 60, n. 3, p. 551-553, May-June 1995.

CHANG, H. C.; ORIEL, P. J. – Bioproduction of Perillyl Alcohol and Related Monoterpenes by Isolates of *Bacillus stearothermophilus*. **Journal of Food Science.** v. 59, n. 3, p. 660-662 and p. 686, May-June 1994.

CHATTERJEE, T. – Biotransformation of Geraniol by *Rhodococcus* sp. Strain GR3. **Biotechnology and Applied Biochemistry.** v. 39, n. 3, p. 303-306, 2004.

CHATTERJEE, T.; BHATTACHARYYA, D. K. – Biotransformation of Limonene by *Pseudomonas putida*. **Applied Microbiology and Biotechnology.** v. 55, n. 5, p. 541-546, May 2001.

CHATTERJEE, T.; DE, B. K.; BHATTACHARYYA, D. K. Microbial Oxidation of α-Pinene to (+)-α-Terpineol by *Candida tropicalis*. **Indian Journal of Chemistry, Section B: Organic Chemistry Including Medicinal Chemistry.** V. 38B, n. 4, p. 515-517, Apr. 1999.

CHEN, S-J., CHENG, C-Y., CHEN, T-L. – Production of an Alkaline Lipase by *Acinobacter radioresistens*. **Journal of Fermentation and Bioengineering.** V. 86, p. 308-312, 1998.

CHEONG, T. K.; ORIEL, P. J. – Cloning and Expression of the Limonene Hydroxylase of *Bacillus stearothermophilus* BR388 and Utilization in Two-Phase Limonene Conversions. **Applied Biochemistry and Biotechnology.** v. 84, p. 903-915, 2000.

CHRISTEN, P.; BRAMORSKY, A.; REVATH, A.; SOCCOL, C. R. – Characterization of Volatile Compounds Produced by *Rhizopus* Strains Grown on Agro-Industrial Solid Wastes. **Bioresource Technology.** V. 71, p. 211-215, 2000.

CHUNG, B.H.; LEE, H.-Y.; LEE, J.S.; YOUNG C.Y.F. – Perillyl Alcohol Inhibits the Expression and Function of the Androgen Receptor in Human Prostate Cancer Cells. **Cancer Letters.** V. 236, p. 222–228, 2006.

COLOCOUSI, A.; SAQUIB K. M.; LEAK, D. J. – Mutants of *Pseudomonas fluorescens* NCIMB 11671 Defective in the Catabolism of α-Pinene. **Applied Microbiology and Biotechnology.** V. 45, p. 822-830, 1996.

CONCEIÇÃO, A. – **Caracterização de Constituintes Nutricionais e Antinutricionais do Resíduo industrial de Laranja (Citrus sinensis L. Osbeck) CV. Hamlim Submetido a**

Diferentes Tipos de Secagens. Lavras: UFLA, 1998 (Tese – Mestrado em Ciência de Alimentos).

CONRAD, H. E.; DUBUS, R.; GUNSALUS, I. C. – An Enzyme System for Cyclic Ketone Lactonization. **Biochemical and Biophysical Research Communications.** V. 6, n. 4, p. 293-297, 1961.

COPPEN, J. J. W., JAMES, D. J., ROBINSON, J. M. AND SUBANSENEE, W. – Variability in Xylem Resin Compositions Amongst Natural Populations of Thai and Filipino *Pinus merkusii* de Vriese. **Flavour and Fragrance Journal.** V. 13, p. 33-39, 1998.

COXON, J. M.; DANSTED, E.; HARTSHORN, M. P. – Allylic Oxidation with Hydrogen Peroxide-Selenium Dioxide: Trans-Pinocarveol. **Organic Syntheses.** V. 56, p. 25-27, 1977.

COXON, J. M.; DANSTED, E.; HARTSHORN, M. P. – *trans*-Pinocarveol from New Zealand Turpentine. **Journal of Chemical and Engineering Data.** V. 15, n. 2, p. 336, 1970.

CROWELL, P. – Prevention and Therapy of Cancer by Dietary Monoterpenes. **Journal of Nutrition.** v. 129, n. 3, p. 775S-778S, 1999.

DA FONSECA, C.O.; LANDEIRO, J. A.; CLARK, S.S.; QUIRICO-SANTOS, T.; CARVALHO, M.G.C.; GATTASS, C.R. – Recent Advances in the Molecular Genetics of Malignant Gliomas Disclose Targets for Antitumor Agent Perillyl Alcohol. **Surgical Neurology**. v. 65, p. S2-S9, 2006a.

DA FONSECA, C.O.; MASINI, M.; FUTURO, D.; CAETANO, R.; GATTASS, C.R.; QUIRICO-SANTOS, T. – Anaplastic Oligodendroglioma Responding Favorably to Intranasal Delivery of Perillyl Alcohol: A Case Report and Literature Review. **Surgical Neurology**. v. 66, n. 6, p. 611-615, 2006b.

DA FONSECA, C.O.; SCHWARTSMANN, G.; FISCHER, J.; NAGEL, J.; FUTURO, D; QUIRICO-SANTOS, T.; GATTASS C.R. – Preliminary Results from a Phase I/II Study of Perillyl Alcohol Intranasal Administration in Adults with Recurrent Malignant Gliomas. **Surgical Neurology**. v. 70, n. 3, p. 259-266, 2008.

DÁVALOS, A., GÓMEZ-CORDOVÉS, C., BARTOLOMÉ B. – Extending Applicability of the Oxygen Radical Absorbance Capacity (ORAC-Fluorescein) Assay. **Journal of Agricultural and Food Chemistry**. V. 52, p. 48 -54, 2004.

DAVIES, D. D.; PATIL, K. D.; UGOCHUKWU, E. N.; TOWERS, G. H. N. – Aliphatic Alcohol Dehydrogenase from Potato Tubers. **Phytochemistry.** V. 12, n. 3, p. 523-530, 1973.

DE CARVALHO, C., C., C., R.; DA FONSECA; M. M. R. – Biotransformations of Terpenes. **Biotechnology Advances.** V. 24, n. 2, p. 134-142, 2006a.

DE CARVALHO, C. C. C. R.; DA FONSECA, M. M. R. – Carvone: Why and how should one bother to produce this terpene. **Food Chemistry.** V. 95, n. 3, p. 413-422, Apr. 2006b.

DE KRAKER, J.-W.; FRANSSEN, M. C. R.; DALM, M. C. F.; DE GROOT, A.; BOUWMEESTER, H. J. Biosynthesis of Germacrene A Carboxylic Acid in Chicory Roots. Demonstration of a Cytochrome P450 (+)-Germacrene A Hydroxylase and $NADP^+$-Dependent Sesquiterpenoid Dehydrogenase(s) Involved in Sesquiterpene Lactone Biosynthesis. **Plant Physiology.** V. 125, p. 1930-1940, 2001.

DE KRAKER, J.-W.; SCHURINK, M.; FRANSSEN, M. C. R.; KONIG, W. A.; DE GROOT, A.; BOUWMEESTER, H. J. Hydroxylation of Sesquiterpenes by Enzymes from Chicory (*Cichorium intybus* L.) Roots. **Tetrahedron.** V. 59, n. 3, p. 409-418, 2003.

DEMYTTENAERE, J. C. R. – Biotransformation of Terpenoids by Microorganisms. In: RAHMAN, A (Ed) **Studies in Natural Products Chemistry**. London: Elsevier, 2001, V. 25F, pp 125-178.

DEMYTTENAERE, J. C. R.; DE POOTER, H. L. – Biotransformation of Citral and Nerol by Spores of *Penicillium digitatum*. **Flavour and Fragrance Journal.** V. 13, n. 3, p. 173-176, 1998.

DEMYTTENAERE, J. C. R.; DE POOTER, H. L. – Biotransformation of Geraniol and Nerol by Spores of *Penicillium italicum*. **Phytochemistry.** V. 41, n. 4, p. 1079-1082, 1996.

DEMYTTENAERE, J. C. R.; VAN BELLEGHEM, K.; DE KIMPE, N. – Biotransformation of (R)-(+)- and (S)-(–)-Limonene by Fungi and the Use of Solid Phase Microextraction for Screening. **Phytochemistry.** v. 57, n. 2, p. 199-208, May 2001.

DEMYTTENAERE, J. C. R.; VANOVERSCHELDE, J.; DE KIMPE, N. – Biotransformation of (R)-(+)- and (S)-(-)-Citronellol by *Aspergillus* sp. and *Penicillium* sp., and the use of Solid-Phase Microextraction for Screening. **Journal of Chromatography, A.** v. 1027, n. 1-2, p. 137-146, 2004.

DEMYTTENAERE, J. C. R.; WILLEMEN, H. M. – Biotransformation of Linalool to Furanoid and Pyranoid Linalool Oxides by *Aspergillus niger*. **Phytochemistry.** v. 47, n. 6, p. 1029-1036, Mar. 1998.

DERFER J. M.; KANE B. J.; YOUNG D. G. – **Preparation of Carvone**. US Patent number US3293301. Dec. 20 1966.

DHAVALIKAR, R. S.; BHATTACHARYYA, P. K. – Microbiological Transformation of Terpenes: Part VIII – Fermentation of Limonene by a Soil Pseudomonad. **Indian Journal of Biochemistry.** v. 3, p. 144-157, Sept. 1966.

DHAVALIKAR, R. S.; RANGACHARI, P. N.; BHATTACHARYYA, P. K. – Microbiological Transformation of Terpenes: Part IX – Pathways of Degradation of Limonene in a Soil Pseudomonad. **Indian Journal of Biochemistry.** v. 3, p. 158-164, Sept. 1966.

DINCALP, H.; IÇLI, S. – Photosynthesis of Rose Oxide by Concentrated Sunlight in the Absence of Singlet Oxygen. **Journal of Photochemistry and Photobiology, A: Chemistry**. V. 141, n. 2-3, p. 147-151, 2001.

DIVYASHREE, M. S.; GEORGE, J., AGRAWAL, R. – Biotransformation of Terpenic Substrates by Resting Cells of *Aspergillus niger* and *Pseudomonas putida* Isolates. **Journal of Food Science and Technology**. V. 43, n. 1, p. 73-76, 2006.

DRACZYŃSKA, B.; CAGARA, Cz.; SIEWIŃSKI, A.; RYMKIEWICZ, A.; ZABŻA, A.; LEUFVÈN, A. – Biotransformation of Pinenes. XVII. Transformation of α- and β-Pinenes by Means of *Armillariella mellea* (Honey Fungus), a Parasite of Woodlands. **Journal of Basic Microbiology.** V. 25, n. 8, p. 487-492, 1985.

DUETZ, W. A.; BOUWMEESTER, H.; VAN BEILEN, J. B.; WITHOLT, B. – Biotransformation of Limonene by Bacteria, Fungi, Yeasts, and Plants. **Applied Microbiology and Biotechnology.** V. 61, n. 4, p. 269-277, May 2003.

DUETZ, W. A.; FJÄLLMAN, A. H. M.; REN, S.; JOURDAT, C; WITHOLT, B. – Biotransformation of d-Limonene to (+) trans-Carveol by Toluene-Grown *Rhodococcus opacus* PWD4 Cells. **Applied and Environmental Microbiology.** V. 67, n. 6, p. 2829-2832, June 2001.

DUETZ, W. A.; VAN BEILEN, J. B.; WITHOLT, B. – Using Proteins in their Natural Environment: Potential and Limitations of Microbial Whole-Cell Hydroxylations in Applied Biocatalysis. **Current Opinion in Biotechnology.** V. 12, p. 419-425, 2001.

DUETZ, W. A.; WITHOLT, B.; JOURDAT, C. – **Process for the Preparation of Perillyl Alcohol.** United States Patent Application n. 20040077063. Apr. 22, 2004.

EISENREICH, W.; ROHDICH, F.; BACHER A. – Deoxyxylulose Phosphate Pathway to Terpenoids. **Trends in Plant Science.** V. 6, n. 2, p. 78-84, Feb. 2001.

EL FIRDOUSSI, L.; BAQQA, A.; ALLAOUD, S.; ALLAL, B. A.; KARIM, A.; CASTANET, Y.; MORTREUX, A. – Selective Palladium-Catalyzed Functionalization of Limonene: Synthetic and Mechanistic Aspects. **Journal of Molecular Catalysis A: Chemical.** V. 135, n. 1, p. 11-22, 1998.

EL-GHORAB, A.; EL-MASSRY, K. F.; SHIBAMOTO, T. – Chemical Composition of the Volatile Extract and Antioxidant Activities of the Volatile and Nonvolatile Extracts of Egyptian Corn Silk (Zea mays L.). **Journal of Agricultural and Food Chemistry.** V. 55, n. 22, p. 9124-9127, 2007.

ERTUĞRUL, S., DÖNMEZ, G., TAKAÇ, S. – Isolation of Lipase Producing *Bacillus* sp. from Olive Mill Wastewater and Improving its Enzyme Activity. **Journal of Hazardous Materials.** V. 149, n. 3, p. 720-724, 2007.

FAO – **Food and Agriculture Organization of the United Nations.** Statistical Database. Disponível em <http://faostat.fao.org/site/567/default.aspx>. Acessado em 03 dez 2008.

FAROOQ, A., CHOUDHARY, M. I., TAHARA, S., RAHMAN, A., BASER, K. H. C.; DEMIRCI, F. – The Microbial Oxidation of (–)-β-Pinene by *Botrytis cinerea*. **Zeitschrift fuer Naturforschung, C: Journal of Biosciences**. V. 57, n. 7-8, p. 686-690, 2002.

FENAROLI, G. – **Fenaroli's Handbook of Flavor Ingredients.** Translated by FURIA, T. E.; BELLANCA, N. Cleveland: The Chemical Rubber Co., 1971. 803 p.

FIGUEIREDO, A. C.; ALMENDRA, M. J.; BARROSO, J. G.; SCHEFFER, J. J. C. – Biotransformation of Monoterpenes and Sesquiterpenes by Cell Suspension Cultures of *Achillea millefolium* L. ssp. *millefolium*. **Biotechnology Letters.** V. 18, n. 8, p. 863-868, 1996.

FLEISCHMANN, P.; STUDER, K.; WINTERHALTER, P. – Partial Purification and Kinetic Characterization of a Carotenoid Cleavage Enzyme from Quince Fruit (*Cydonia oblonga*). **Journal of Agricultural and Food Chemistry.** V. 50, p. 1677-1680, 2002.

FLEISCHMANN, P.; WATANABE, N.; WINTERHALTER, P. – Enzymatic Carotenoid Cleavage in Star Fruit (*Averrhoa carambola*). **Phytochemistry.** V. 63, n. 2, p. 131-137, 2003.

FONTANILLE, P. – **Biotransformation de α-pinene oxyde en cis-2-methyl-5-isopropylhexa-2,5-dienal (isonovalal) par *Pseudomonas rhodesiae* CIP 107491**. Clermont-Ferrand, France: Université Blaise Pascal, 2002 (Tese – Doutorado)

FONTANILLE, P.; LARROCHE, C. – Optimization of Isonovalal Production from α-Pinene Oxide Using Permeabilized Cells of *Pseudomonas rhodesiae* CIP107491. **Applied Microbiology and Biotechnology.** V. 60, 534-540, 2003.

FONTANILLE, P., LE FLÈCHE, A.; LARROCHE, C. – *Pseudomonas rhodesiae* PF1: A New and Efficient Biocatalyst for Production of Isonovalal from α-Pinene Oxide. **Biocatalysis and Biotransformations.** V. 20, p. 413-421, 2002.

FÖRSTER-FROMME, K., HÖSCHLE, B., MACK, C., BOTT, M., ARMBRUSTER, W., JENDROSSEK, D. – Identification of Genes and Groteins Necessary for Catabolism of Acyclic Terpenes and Leucine/Isovalerate in *Pseudomonas aeruginosa*. **Applied and Environmental Microbiology.** V. 72, p. 4819-4828, 2006.

FRANCO, M. R. B. – **Aroma e Sabor dos Alimentos:** Temas Atuais. São Paulo: Livraria Varela. 2004. 246 p.

FURUSAWA, M.; HASHIMOTO, T.; NOMA, Y.; ASAKAWA Y. – Biotransformation of Citrus Aromatics Nootkatone and Valencene by Microorganisms. **Chemical and Pharmaceutical Bulletin.** V. 53, n.11, p.1423-1429, 2005.

GERDES, R.; BARTELS, O.; SCHNEIDER, G.; WÖHRLE, D.; SCHULZ-EKLOFF, G. – Photooxidations of Phenol, Cyclopentadiene and Citronellol with Photosensitizers Ionically Bound at a Polymeric Ion Exchanger. **Polymers for Advanced Technologies**. V. 12, n. 3-4, p. 152-160, 2001.

GERSHENZON, J.; DUDAREVA, N. – The Function of Terpene Natural Products in the Natural World. **Nature Chemical Biology**. V. 3**,** n. 7, p. 408-414, 2007.

GERSON, D. F. – The Biophysics of Microbial Surfactants: Growth on Insoluble Substrates In: KOSARIC, N. (Ed.) **Biosurfactants: Production, Properties, Applications**. New York: Marcel Dekker Inc., 1993, ch. 10, pp. 269-286.

GIBBON, G. H.; PIRT, S. J. – The Degradation of α-Pinene by *Pseudomonas* PX1. **FEBS Letters.** V. 18, n. 1, p. 103-105, Oct. 1971.

GIRI, A.; DHINGRA, V.; GIRI, C. C.; SINGH, A.; WARD, O. P.; NARASU, M. L. – Biotransformation Using Plant Cells, Organ Cultures and Enzyme Systems: Current Trends and Future Prospects. **Biotechnology Advances.** V. 19, p. 175-199, 2001.

GOMES, M. F. T.; ANTUNES, O. A. C. – Oxidation of Limonene Catalyzed by Mn^{III}(Salen)Cl.H_2O. **Catalysis Letters.** V. 38, p. 133-134, 1996a.

GOMES, M. F. T.; ANTUNES, O.A.C. – Oxidation of α and β-Pinene Catalyzed by Mn^{III}(Salen)Cl.H_2O. **Catalysis Letters.** V. 42, p. 213-215, 1996b.

GONÇALVES, J. A.; BUENO, A. C.; GUSEVSKAYA, E. V. – Palladium-Catalyzed Oxidation of Monoterpenes: Highly Selective Syntheses of Allylic Ethers from Limonene. **Journal of Molecular Catalysis A: Chemical.** V. 252, n. 1-2, p. 5-11, 2006.

GONÇALVES, J. A.; SILVA, M. J.; PILO-VELOSO, D.; HOWARTH, O. W.; GUSEVSKAYA, E. V. – Palladium Catalyzed Oxidation of Monoterpenes: NMR Study of Palladium(II)-Monoterpene Interactions. **Journal of Organometallic Chemistry.** V. 690, n. 12, p. 2996-3003, 2005.

GOTOR-FERNÁNDEZ, V., BRIEVA, R., GOTOR, V. – Lipases: Useful biocatalysts for the preparation of pharmaceuticals. **Journal of Molecular Catalysis B: Enzymatic**. V. 40, p. 111-120, 2006.

GRAYSON, D. H. – Monoterpenoids. **Natural Product Reports.** V. 17, n. 4, p. 385-419, Aug 2000.

GRIFFIN, S. G.; WHYLLIE, S. G.; MARKHAM, J. L.; LEACH, D. N. – The Role of Structure and Molecular Properties of Terpenoids in Determining their Antimicrobial Activity. **Flavour and Fragrance Journal.** V. 14, n. 5, p. 322-332, 1999.

GRIFFITHS, E. T.; BOCIEK, S. M.; HARRIES, P. C.; JEFFCOAT, R.; SISSONS, D. J.; TRUDGILL, P. W. – Bacterial Metabolism of α-Pinene: Pathway from α-Pinene Oxide to Acyclic Metabolites in *Nocardia* sp. Strain P18.3. **Journal of Bacteriology.** V. 169, n. 11, p. 4972-4979, Nov. 1987a.

GRIFFITHS, E. T., HARRIES, P. C., JEFFCOAT, R.; TRUDGILL, P. W. –Purification and properties of α-pinene oxide lyase from *Norcadia* sp. strain P18.3. **Journal of Bacteriology.** V. 169, n. 11, p. 4980-4983, 1987b.

GRIVEL, F.; LARROCHE, C. – Phase Transfer and Biocatalyst Behaviour during Biotransformation of β-Ionone in a Two-phase Liquid System by Immobilised *Aspergillus niger*. **Biochemical Engineering Journal.** V. 7 , n. 1, p. 27-34, 2001.

GRIVEL, F.; LARROCHE, C.; GROS J. B. – Determination of the Reaction Yield during Biotransformation of the Volatile and Chemically Unstable Compound β-Ionone by *Aspergillus niger*. **Biotechnology Progress.** V. 15, n. 4, p. 697-705, 1999.

GROVES, J. T.; SUBRAMANIAN, D. V. – Hydroxylation by Cytochrome P-450 and Metalloporphyrin Models. Evidence for Allylic Rearrangement. **Journal of the American Chemical Society.** V. 106, n. 7, p. 2177-2181, 1984.

GURAJEYALAKSHMI, G.; ORIEL, P. – Isolation of Phenol-Degrading *Bacillus stearothermophilus* and Partial Characterization of the Phenol Hydroxylase. **Applied and Environmental Microbiology.** V. 55, n. 2, 500-502, 1989.

GUSEVSKAYA, E. V.; GONSALVES, J. A. – Palladium(II) Catalyzed Oxidation of Naturally Occurring Terpenes with Dioxygen. **Journal of Molecular Catalysis A: Chemical.** V. 121, n. 2-3, p. 131-137, 1997.

GUSEVSKAYA, E. V.; ROBLES-DUTENHEFNER, P. A.; FERREIRA, V. M. S. – Palladium-Catalyzed Oxidation of Bicyclic Monoterpenes by Hydrogen Peroxide. **Applied Catalysis, A: General.** V. 174, n. 1-2, p. 177-186, 1998.

HAGEDORN, S.; KAPHAMMER, B. – Microbial Biocatalysis in the Generation of Flavor and Fragrance Chemicals. **Annual Review of Microbiology.** V. 48, p. 773-800, 1994.

HAMADA, H.; FUCHIKAMI, Y.; IKEMATSU, Y.; HIRATA, T.; WILLIAMS, H. J.; SCOTT A. I. – Hydroxylation of Piperitone by Cell Suspension Cultures of *Catharanthus roseus*. **Phytochemistry.** V. 37, n. 4, p. 1037-1038, 1994.

HAMADA, H.; KONDO, Y.; ISHIHARA, K.; NAKAJIMA, N.; HAMADA, H.; KURIHARA, R.; HIRATA, T. – Stereoselective Biotransformation of Limonene and Limonene Oxide by Cyanobacterium, *Synechococcus* sp. PCC 7942. **Journal of Bioscience and Bioengineering**. V. 96, n. 6, p.581–584, 2003.

HARDER, J.; PROBIAN, C. – Microbial Degradation of Monoterpenes in the Absence of Molecular Oxygen. **Applied and Environmental Microbiology.** V. 61, n. 11, p. 3804-3808, Nov. 1995.

HATANAKA, A.; SEKIYA, J.; KAJIWARA, T. – Subunit Composition of Alcohol Dehydrogenase from *Thea sinensis* Seeds and its Substrate Specificity for Monoterpenes. **Phytochemistry.** V. 15, n. 4, p. 487-488, 1976.

HAUDENSCHILD, C.; SCHALK, M.; KRAP, F.; CROTEAU, R. – Functional Expression of Regiospecific Cytochrome P450 Limonene Hydroxylases from Mint (*Mentha* spp.) in *Escherichia coli* and *Saccharomyces cerevisae*. **Archives of Biochemistry and Biophysics.** v. 379, n. 1, p. 127-136, Jul. 2000.

HIRATA, T.; IKEDA, Y.; IZUMI, S.; SHIMODA, K.; HAMADA, H.; KAWAMURA, T. – Introduction of Oxigenated Functional Groups into 3-Carene and 2-Pinene by Cultured Cells. **Phytochemistry.** V. 37, n. 2, p. 401-403, 1994.

HIRSJARVI, P. – Selenium Dioxide Oxidation of Camphene. **Suomen Kemistilehti B.** V. 29B, p. 145-146, 1956.

HIRSJARVI, P.; HIRSJARVI, V. P. – Camphenilanaldehyde Enol Acetate from Camphene by Selenium Dioxide Oxidation. **Suomen Kemistilehti B.** V. 38, n. 12, p. 290, 1965.

HIRSJARVI, P.; HIRSJARVI, M.; KAILA, J. O. W. – Selenium Dioxide Oxidation of Camphene. **Suomen Kemistilehti B.** V. 30B, p. 72-73, 1957.

HORIKOSHI, K. Discovering Novel Bacteria, with an Eye to Biotechnological Application. **Current Opinion in Biotechnology**. V. 6, n. 3, p. 292-297, 1995.

HUANG, D.; OU, B.; PRIOR, R. L. – The Chemistry Behind Antioxidant Capacity Assays. **Journal of Agricultural and Food Chemistry.** V. 53, n. 6, p. 1841-1856, 2005.

INOUE, A.; HORIKOSHI, K. – A *Pseudomonas* Thrives in High Concentrations of Toluene. **Nature.** V. 338, p. 264-266, March 1989.

ISHIDA, T. – Biotransformation of Terpenoids by Mammals, Microorganisms, and Plant-Cultured Cells. **Chemistry and Biodiversity.** V. 2, n. 5, p. 569-590, May 2005.

JANSSENS, L.; DE POOTER, H. L.; SCHAMP, N. M.; VANDAMME, E. J. – Production of Flavours by Microorganisms. **Process Biochemistry.** V. 27, p. 195-215, 1992.

JIN, K.-S., JUN, M.; PARK, M.-J.; OK, S.; JEONG, J.-H.; KANG, H.-S.; JO, W.-K.; LIM, H.-J.; JEONG, W.-S. – Promises and Risks of Unsaturated Volatile Organic Compounds: Limonene, Pinene, and Isoprene. **Food Science and Biotechnology.** V. 17; n. 3; p. 447-456, 2008.

JÖNSSON, B.; LINDMAN, B.; HOLMBERG, K.; KRONBERG, B. – Surfactant Micellization. In: JÖNSSON B, LINDMAN B, HOLMBERG K, KRONBERG B (Eds.). **Surfactants and Polymers in Aqueous Solution.** New York: John Wiley & Sons, 1998, ch. 2, pp. 33-60.

JOSHEL, L. M.; PALKIN, S. – Oxidation of β-Pinene with Selenium Dioxide. **Journal of the American Chemical Society**. V. 64**,** p. 1008-1009, 1942.

JUN, M.; JEONG, W.-S.; HO, C.-T. – Health Promoting Properties of Natural Flavor Substances. **Food Science and Biotechnology.** V. 15, n. 3, p. 329-335, 2006.

KAMIŃSKA, J.; MARKOWICZ, L.; STOŁOWSKA, J.; GÒRA, J. – Biotransformation of Citronellol by Means of Horseradish Peroxidase. **Enzyme and Microbial Technology.** v. 11; p. 436-438; July 1989.

KARP, F.; MIHALIAK C. A.; HARRIS, J. L.; CROTEAU, R. – Monoterpene Biosynthesis: Specificity of the Hydroxylations of (–)-Limonene by Enzyme Preparations from Peppermint (*Mentha piperita*), Spearmint (*Mentha spicata*), and Perilla (*Perilla frutescens*) Leaves. **Archives of Biochemistry and Biophysics.** v. 276, n. 1, p. 219-226, Jan. 1990.

KASPERA, R.; KRINGS, U.; BERGER, R. G. – Microbial Terpene Biotransformation. In: LARROCHE, C., PANDEY, A.; DUSSAP C. G (Eds.) **Current Topics on Bioprocesses in Food Industry.** New Delhi: Asiatech Publisher, Inc., 2006, pp.54-69.

KIESLICH, K. Introduction. In: REHM H. -J.; REED G. (Eds.) **Biotechnology.** Weinheim: Verlag Chemie GmbH, Vol. 6a, 1984, pp. 1-4.

KING, A. J.; DICKINSON, J. R. – Biotransformation of Hop Aroma Terpenoids by Ale and Lager Yeasts. **FEMS Yeast Research.** V.3, n. 1, p. 53-62, 2003.

KJONAAS, R.; MARTINKUS-TAYLOR, C.; CROTEAU, R. – Metabolism of Monoterpenes: Conversion of *l*-Menthone to *l*-Menthol and *d*-Neomenthol by Stereospecific Dehydrogenases from Peppermint (*Mentha piperita*) Leaves. **Plant Physiology.** V. 69, n. 5, p. 1013-1017, 1982.

KJONAAS, R. B.; VENKATACHALAM, K. V.; CROTEAU, R. – Metabolism of Monoterpenes: Oxidation of Isopiperitenol to Isopiperitenone, and Subsequent Isomerization to Piperitenone by Soluble Enzyme Preparations from Peppermint (*Mentha piperita*) Leaves. **Archives of Biochemistry and Biophysics.** V. 238, n. 1, p. 49-60, 1985.

KRAIDMAN, G.; MUKHERJEE, B. B.; HILL, J. D. – Conversion of D-Limonene into an Optically Active Isomer of α-Terpineol by a *Cladosporium* Species. **Bacteriological Proceedings.** V. 69, p. 63, 1969.

KRASNOBAJEW, V.; HELMLINGER, D. – Fermentation of Fragrances: Biotranformation of β-ionone by *Lasiodiplodia theobromae*. **Helvetica Chimica Acta.** V. 65, p. 1590-1601, 1982.

KRINGS, U.; BERGER, R.G. – Biotechnological Production of Flavours and Fragrances. **Appied Microbiology and Biotechnology**. V. 49, n. 1, p. 1-8, 1998.

KRINGS, U.; HARDEBUSCH, B.; ALBERT, D.; BERGER, R. G.; MAROSTICA JR., M.; PASTORE, G. M. – Odor-Active Alcohols from the Fungal Transformation of α-Farnesene. **Journal of Agricultural and Food Chemistry.** V. 54, p. 9079-9084, 2006.

KUTTUVA, S. G.; RESTREPO, A. S.; JU. L.-K. – Evaluation of Different Organic Phases for Water-in-Oil Xanthan Fermentation. **Appied Microbiology and Biotechnology.** V. 64, p. 340–345, 2004.

LAGUERRE, M.; LACOMTE, J.; VILLENEUVE, P. – Evaluation of the Ability of Antioxidants to Counteract Lipid Oxidation: Existing Methods, New Trends and Challenges. **Progress in Lipid Research.** V. 46, p. 244-282, 2007.

LANGENHEIM, J. H. – Higher Plant Terpenoids: A Phytocentric Overview of their Ecological Roles. **Journal of Chemical Ecology.** V. 20, n. 6, June 1994.

LAROCHE, C.; FONTANILLE, P.; LARROCHE, C. – Purification of α-Pinene Oxide Lyase from *Pseudomonas rhodesiae* CIP 107491. In: LARROCHE, C., PANDEY, A.; DUSSAP C. G (Eds.) **Current topics on bioprocesses in food industry.** New Delhi: Asiatech Publisher, Inc., 2006, pp. 98-108.

LARROCHE, C.; CREULY, C.; GROS, J. B. – Fed-Batch Biotransformation of β-Ionone by *Aspergillus niger*. **Applied Microbiology and Biotechnology.** V. 43, n. 2, p. 222-227, 1995.

LEGOY, M. D.; KIM, H. S.; THOMAS, D. – Use of Alcohol Dehydrogenase for Flavour Aldehyde Production. **Process Biochemistry.** V. 20, n. 5, p. 145-148, Oct. 1985.

LEUENBERGER, H. G. W. – Biotransformation – A Useful Tool in Organic Chemistry. **Pure and Applied Chemistry.** V. 62, n. 4, p. 753-768, 1990.

LEUENBERGER, H. G. W. Methodology. In REHM H. -J.; REED G. (Eds.) **Biotechnology.** Weinheim: Verlag Chemie GmbH, Vol. 6a, 1984, pp 5-29.

LI, N. – Appraising on Catalyst of α-Pinene Oxidation Using Fixed Bed Reactor. **Guilin Gongxueyuan Xuebao**. V. 20, n. 3, p. 270-272, 2000a.

LI, N. – Study on Kinetics of Catalytic Oxidation Reaction of α-Pinene to Myrtenal. **Guilin Gongxueyuan Xuebao**. V. 20, n. 4, p. 406-408, 2000b.

LIMA, L. F.; CARDOZO-FILHO, L.; ARROYO, P. A.; MÁRQUEZ-ALVAREZ, H.; ANTUNES, O. A. C. – Metal(Salen)-Catalyzed Oxidation of Limonene in Supercritical CO_2. **Reaction Kinetics and Catalysis Letters.** V. 84, n. 1, p. 69-77, 2005.

LIMA, L. F.; CORRAZA, M. L.; CARDOZO-FILHO, L.; MÁRQUEZ-ALVAREZ, H.; ANTUNES, O. A. C. – Oxidation of Limonene Catalyzed by Metal(Salen) Complexes. **Brazilian Journal of Chemical Engineering.** V. 23, n. 1, p. 83-92, 2006.

LIMA, V. M. G., KRIEGER, N., MITCHELL, D. .A., FONTANA, J. D. – Activity and Stability of a Crude Lipase from *Penicillium aurantiogriseum* in Aqueous Media and Organic Solvents. **Biochemical Engineering Journal**. V. 18, p. 65-71, 2004.

LIN, S- F., CHIOU, C- M., TSAI, Y- C. – Effect of Triton X-100 on Alkaline Lipase Production by *Pseudomonas pseudoalcaligenes* F-111. **Biotechnology Letters**. V. 17, n. 9, p. 956-962, 1995.

LIN, Q.; LI, X.; DENG, S. – Preparation of Myrtenal by Oxidation of α-Pinene. **Huaxue Shijie.** V. 31, n. 7, p. 299-300, 1990. Abstract.

LINARES, D. – **Etudes sur la Voie de Dégradation de l'α-Pinène Chez *Pseudomonas rhodesiae* en Milieu Biphasique Liquide-Liquide.** Clermont-Ferrand, France: Université Blaise Pascal, 2008 (Tese – Doutorado em Ciências da Vida e da Saúde).

LINARES, D.; MARTINEZ, D.; FONTANILLE, P.; LARROCHE, C. – Production of *trans*-2-methyl-5-isopropylhexa-2,5-dienoic Acid by *Pseudomonas rhodesiae* CIP 107491. **Bioresource Technology**. V. 99, n. 11, p. 4590-4596, 2008.

LINDMARK-HENRIKSSON, M.; ISAKSSON, D.; SJÖDIN, K.; HÖGBERG, H.-E.; VANĚK, T.; VALTEROVÁ, I. – Transformation of α-Pinene Using *Picea abies* Suspension Culture. **Journal of Natural Products.** V. 66, n. 3, p. 337-343, 2003.

LINDMARK-HENRIKSSON, M.; ISAKSSON, D.; VANĚK, T.; VALTEROVÁ, I.; HÖGBERG, H.-E; SJÖDIN, K. – Transformation of Terpenes Using a *Picea abies* Suspension Culture. **Journal of Biotechnology.** V. 107, n. 2, p. 173-184, 2004.

LIU, J.-L; DENG, S.-H.; CHENG, X.-L. – Preparation of Perillartine from Turpentine. **Journal of Guangdong University of Technology.** V. 17, n. 4, p. 75-77, 2000.

LOMASCOLO, A.; STENTELAIRE, C.; ASTHER, M.; LESAGE-MEESSEN, L. – Basidiomycetes as New Biotechnological Tools to Generate Natural Aromatic Flavors for the Food Industry. **Trends in Biotechnology.** V. 17, n. 7, p. 282-289, 1999.

LOPES-LUTZ, D.; ALVIANO, D. S.; ALVIANO, C. S.; KOLODZIEJCZYK, P. P. – Screening of Chemical Composition, Antimicrobial and Antioxidant Activities of *Artemisia* essential oils. **Phytochemistry**. V. 69, n. 8, p. 1732-1738, May 2008.

LUPIEN, S.; KARP, F.; WILDUNG, M.; CROTEAU, R. - Regiospecific Cytochrome P450 Limonene Hydroxylases from Mint (*Mentha*) Species: cDNA Isolation, Characterization, and Functional Expression of (–)-4S-Limonene-3-Hydroxylase and (–)-4S-Limonene-6-Hydroxylase. **Archives of Biochemistry and Biophysics**. v. 368, n. 1, p. 181-192, Aug. 1999.

LUTZ-WAHL, S.; FISCHER, P.; SCHMIDT-DANNERT, C.; WOHLLEBEN, W.; HAUER, H.; SCHMID, R. D. – Stereo- and Regioselective Hydroxilation of α-Ionone by

Streptomyces Strains. **Applied and Environmental Microbiology.** V. 64, n. 10, p. 3878-3881, Oct. 1998.

MACEDO, G. A., LOZANO, M. M. S., PASTORE, G. M. – Enzymatic Synthesis of Short Chain Citronellyl Esters by a New Lipase from *Rhizopus* sp. **Eletronic Journal of Biotechnology**. V. 6, n. 1, p. 72-75, 2003.

MAGALHAES T. M.; KOKETSU M.; WILBERG V. C. – **Processo de Obtenção de *l*-Carvona a Partir de *d*-Limoneno**. Brazilian Patent number BR8200508. Sep. 27 1983.

MAIA, M. M. D., HEASLEY, A., CAMARGO DE MORAIS, M. M., MELO, E. H. M., MORAIS JR., M. A., LEDINGHAM, W. M., LIMA FILHO, J. L. – Effect of Culture Conditions on Lipase Production by *Fusarium solani* in Batch Fermentation. **Bioresource Technology**. V. 76, p. 23-27, 2001.

MAKSIMCHUK, N. V.; MELGUNOV, M. S.; MROWIEC-BIALON, J.; JARZEBSKI, A. B.; KHOLDEEVA, O. A. – H_2O_2-Based Allylic Oxidation of α-Pinene over Different Single Site Catalysts. **Journal of Catalysis.** V. 235, n. 1, p. 175-183, 2005.

MALDONADO-ROBLEDO, G.; RODRIGUEZ-BUSTAMANTE, E.; SANCHEZ-CONTRERAS, A.; RODRIGUEZ-SANOJA, R.; SANCHEZ, S. – Production of tobacco aroma from lutein. Specific role of the microorganisms involved in the process. **Applied Microbiology and Biotechnology.** V. 62, n. 5-6, p. 484-488, 2003.

MAPA – **Ministério da Agricultura, Pecuária e Abastecimento.** Agronegócio Brasileiro: Uma Oportunidade de Investimentos. Disponível em <http://www.agricultura.gov.br/portal/page?_pageid=33,968707&_dad=portal&_schema=PORTAL> Acesso em 03 dez 2008.

MARÓSTICA JR., M. R.; PASTORE, G. M. – Biotransformation of Citronellol in Rose-Oxide using Cassava Wastewater as a Medium. **Ciência e Tecnologia de Alimentos.** V. 26, n. 3, p. 690-696, Jul-Set. 2006.

MARÓSTICA JR., M. R.; PASTORE, G. M. – Biotransformation of Limonene: a Review of the Main Metabolic Pathways. **Química Nova.** V. 30, n. 2, p. 382-387, Mar-Apr. 2007a.

MARÓSTICA JR., M. R.; PASTORE, G. M. – Production of *R*-(+)-α-Terpineol by the Biotransformation of Limonene from Orange Essential Oil, using Cassava Waste Water as Medium. **Food Science.** V. 101, p. 345-350, 2007b.

MARS, A. E.; GORISSEN, J. P. L.; VAN DEN BELD, I.; EGGINK, G. – Bioconversion of Limonene to Increased Concentrations of Perillic Acid by *Pseudomonas putida* GS1 in a Fed-batch Reactor. **Applied Microbiology and Biotechnology.** v. 56, n. 1-2, p. 101-107, July 2001.

MARTINS, R. R. L.; NEVES, M. G. M. S.; SILVESTRE, A. J. D.; SILVA, A. M. S.; CAVALEIRO, J. A. S. – Oxidation of Aromatic Monoterpenes With Hydrogen Peroxide Catalysed by Mn(III) Porphyrin Complexes. **Journal of Molecular Catalysis A: Chemical.** V. 137; n. 1–3; p. 41–47; Jan 1999.

MARTINS, R. R. L.; NEVES, M. G. M. S.; SILVESTRE, A. J. D.; SIMÕES, M. M. Q.; SILVA, A. M. S.; TOMÉ, A. C.; CAVALEIRO, J. A. S.; TAGLIATESA, P.; CRESTINI, C. – Oxidation of Unsaturated Monoterpenes With Hydrogen Peroxide Catalysed by Manganese(III) Porphyrin Complexes. **Journal of Molecular Catalysis A: Chemical.** V. 172; n. 1–2; p. 33–42; July 2001.

MATTHEWS, R.F.; BRADDOCK R.J. – Recovery and Application of Essential Oils from Oranges. **Food Technology**. V. 41, n. 1, p. 57-61, 1987.

MAZZARO, D. – Orange Oil, *D*-limonene Market Unsettled Due to Brazil Delays. **Chemical Market Reporter**. V. 258, n. 4, p. 18, 2000.

MELO, L. L. M. M., PASTORE, G. M., MACEDO, G. A. – Optimized Synthesis of Citronellyl Flavour Esters Using Free and Immobilized Lipase from *Rhizopus* sp. **Process Biochemistry**. V. 40, p. 3181-3185, 2005.

MENÉNDEZ, P.; GARCÍA, C.; RODRÍGUEZ, P.; MOYNA, P.; HEINZEN, H. – Enzymatic Systems Involved in D-limonene Biooxidation. **Brazilian Archives of Biology and Technology.** v. 45, n. 2, p. 111-114, June 2002.

MEYER, S.; TIETZE, D.; RAU, S.; SCHÄFER, B.; KREISEL, G. – Photosensitized Oxidation of Citronellol in Microreactors. **Journal of Photochemistry and Photobiology, A: Chemistry**. V. 186, n. 2-3, p. 248-253, 2007.

MIKAMI, Y.; FUKUNAGA, Y.; ARITA, M.; KISAKI T. – Microbial Transformation of *β*-Ionone and *β*-Methylionone. **Applied and Environmental Microbiology.** V. 41, n. 3, p. 610-617, March 1981.

MIYAZAWA, M.; KUMAGAE, S. – Biotransformation of (*R*)- and (*S*)-Terpinen-4-ol by the Larvae of Common Cutworm (*Spodoptera litura*). **Journal of Agricultural and Food Chemistry.** V. 49, n. 9; p. 4312-4314, 2001.

MIYAZAWA, M.; KUMAGAE, S.; KAMEOKA, H. – Biotransformation of (+)- and (–)-Menthol by the Larvae of Common Cutworm (*Spodoptera litura*). **Journal of Agricultural and Food Chemistry.** V. 47, n. 9; p. 3938-3940, 1999.

MIYAZAWA, M.; MIYAMOTO, T. – Biotransformation of (1*R*)-(+)- and (1*S*)-(–)-Camphor by the Larvae of Common Cutworm (*Spodoptera litura*). **Journal of Molecular Catalysis B: Enzymatic.** V. 27, n. 2-3; p. 83-89, Feb. 2004.

MIYAZAWA, M.; MURATA, T. – Biotransformation of β-Mircene by the Larvae of Common Cutworm (*Spodoptera litura*). **Journal of Agricultural and Food Chemistry.** V. 48, n. 2; p. 123-125, 2000.

MIYAZAWA, M.; OHSAWA, M. – Biotransformation of α-Terpineol by the Larvae of Common Cutworm (*Spodoptera litura*). **Journal of Agricultural and Food Chemistry.** V. 50, n. 17; p. 4916-4918, 2002.

MIYAZAWA, M.; WADA, T.; KAMEOKA, H. – Biotransformation of (+)- and (–)-Limonene by the Larvae of Common Cutworm (*Spodoptera litura*). **Journal of Agricultural and Food Chemistry.** V. 46, n. 1; p. 300-303, 1998.

MIYAZAWA, M.; WADA, T.; KAMEOKA, H. – Biotransformation of α-Terpinene in Common Cutworm Larvae (*Spodoptera litura* Fabricius) **Journal of Agricultural and Food Chemistry.** V. 44, n. 9; p. 2889-2893, 1996.

MOGHADAM, M.; TANGESTANINEJAD, S.; HABIBI, M. H.; MIRKHANI, V. – A Convenient Preparation of Polymer-Supported Manganese Porphyrin and Its Use as Hydrocarbon Monooxygenation Catalyst. **Journal of Molecular Catalysis A: Chemical.** V. 217; n. 1–2; p. 9–12; Aug. 2004.

MONTEIRO, J. L. F.; VELOSO, C. O. – Catalytic Conversion of Terpenes Into Fine Chemicals. **Topics in Cataysis.** V. 27, n. 1-4, p. 169-180, 2004.

MUKHERJEE, B. B.; KRAIDMAN, G.; HILL, I. D. – Synthesis of Glycols by Microbial Transformations of Some Monocyclic Terpenes. **Applied Microbiology.** v. 25, n. 3, p. 447-453, Mar. 1973.

MULDER, A. J.; VAN HELDEN, J. – **Preparation of Carvone**. British Patent Application number GB2008573. June 6 1979.

MULLER, M.; DIRLAM, K.; WENK, H. H.; BERGER, R. G.; KRINGS, U.; KASPERA, R. – **Method for the production of flavor-active terpenes**. US Patent Application n. 20070172934. July 26, 2007.

NAKAI M.; HARADA K.; NISHIMURA M. – **Preparation of Carvone**. Japanese Patent number JP53111038. Sept. 28 1978.

NARUSHIMA, H.; OMORI, T.; MINODA, Y. – Microbial Transformation of α-Pinene. **European Journal of Applied Microbiology and Biotechnology.** V. 16, n. 4, p. 174-178, 1982.

NATARAJAN, M.R.; LU, Z.; ORIEL, P. – Cloning and Expression of a Pathway for Benzene and Toluene from *Bacillus stearothermophilus*. **Biodegradation.** V. 5, n. 2, p. 77-82, 1994.

NIELSEN, D. R.; DAUGULIS, A. J.; MCLELLAN P. J. – A Novel Method of Simulating Oxygen Mass Transfer in Two-Phase Partitioning Bioreactors. **Biotechnology and Bioengineering.** V. 83, n. 6, p. 735-742, 2003.

NOMA, Y.; AKEHI, E.; MIKI, N.; ASAKAWA, Y. – Biotransformation of Terpene Aldehydes, Aromatic Aldehydes and Related Compounds by *Dunaliella tertiolecta.* **Phytochemistry**. V. 31, n. 2, p. 515-517, Feb. 1992.

NOMA, Y.; YAMASAKI, S.; ASAKAWA, Y. – Biotransformation of Limonene and Related Compounds by *Aspergillus cellulosae*. **Phytochemistry.** v. 31, n. 8, p. 2725-2727, 1992.

OELGEMÖLLER, M.; JUNG, C.; MATTAY J. – Green Ohotochemistry: Oroduction of Fine Chemicals with Sunlight. **Pure and Applied Chemistry**. V. 79, n. 11, p. 1939-1947, 2007.

OELGEMÖLLER, M.; JUNG, C.; ORTNER, J.; MATTAY, J.; ZIMMERMANN E. **–** Green Photochemistry: Solar Photooxygenations with Medium Concentrated Sunlight. **Green Chemistry**. V. 7, n. 1, p. 35-38, 2005.

ONKEN, J.; BERGER, R. G. – Biotransformation of Citronellol by the basidiomycete *Cystoderma carcharias* in an Aerated-Membrane Bioreactor. **Applied Microbiology and Biotechnology.** V. 51. p. 158-163, 1999a.

ONKEN, J.; BERGER, R. G. – Effects of *R*-(+)-Limonene on Submerged Cultures of the Terpene Transforming Basidiomycete *Pleurotus saidus*. **Journal of Biotechnology**. V. 69, n. 2-3, p. 163-168, 1999b.

PANDEY, A.; SOCCOL, C. R.; NIGAM P.; BRAND, D.; MOHAN, R.; ROUSSOS, S. – Biotechnological Potential of Coffee Pulp and Coffee Husk for Bioprocess. **Biochemical Engineering Journal.** V. 6, n. 2, p. 153-162, Oct. 2000a.

PANDEY, A.; SOCCOL, C.R.; NIGAM, P.; SOCCOL, V.T. – Biotechnological Potential of Agro-Industrial Residues. I. Sugarcane Bagasse. **Bioresource Technology**. V. 74, n. 1, p. 69–80, 2000b.

PANDEY, A.; SOCCOL, C.R.; NIGAM, P.; SOCCOL, V.T.; VANDENBERGHE L.P.S.; MOHAN, R. – Biotechnological Potential of Agro-Industrial Residues. II. Cassava Bagasse. **Bioresource Technology**. V. 74, n. 1, p. 81–87, 2000c.

PINHEIRO, L.; MARSAIOLI, A. T. – Microbial Monooxygenases Applied to Fragance Compounds. **Journal of Molecular catalysis B: Enzymatic**. V. 44, p. 78-86. 2007.

PIO, T. F.; MACEDO, G. A. Optimizing the Production of Cutinase by *Fusarium oxysporum* Using Response Surface Methodology. **Enzyme and Microbial Technology**. V. 41, n. 5, p. 613-619, 2007.

PISANCHYN, J.; SIFNIADES, S.; FUHRMANN, R.; KOFF, F. W. – **Nitrosochlorination of Cyclo-Olefins**. US Patent number US3931343. Jan. 6 1976.

POHLMANN, B.; SCHARF, H.-D.; JAROLIMEK, U.; MAUERMANN, P. – Photochemical Production of Fine Chemicals with Concentrated Sunlight. **Solar Energy**. V. 61, n. 3, p. 159-168, 1997.

PRAZERES, J. N. – **Produção, Purificação e Caracterização da Lipase Alcalina de *Fusarium oxysporum*.** Campinas: Unicamp, 2006 (Tese – Doutorado em Ciência de Alimentos).

PRAZERES, J. N.; CRUZ, J. A. B.; PASTORE, G. M. – Characterization of Alkaline Lipase from *Fusarium oxysporum* and the Effect of Different Surfactants and Detergents on the Enzyme Activity. **Brazilian Journal of Microbiology.** V. 37, n. 4, p. 505-509, Out./Dez. 2006.

PREMA, B. R.; BHATTACHARYYA, P. K. – Microbiological Transformation of Terpenes. II. Transformations of α-Pinene. **Applied Microbiology.** V. 10, p. 524-528, 1962a.

PREMA, B. R.; BHATTACHARYYA, P. K. – Microbiological Transformation of Terpenes. III. Transformations of some Mono- and Sesqui-Terpenes. **Applied Microbiology.** V. 10, p. 529-531, 1962b.

RAJMOHAN, S.; DODD, C. E. R.; WAITES, W. M. – Enzymes from Isolates of *Pseudomonas fluorescens* Involved in Food Spoilage. **Journal of Applied Microbiology**. V. 93, p. 205-213, 2002.

RAO, S. C. V.; RAO, R.; AGRAWAL, R. – Enhanced Production of Verbenol, a Highly Valued Food Flavourant, by an Intergeneric Fusant Strain of *Aspergillus niger* and *Penicillium digitatum*. **Biotechnology and Applied Biochemistry.** V. 37, n. 2, p. 145-147, 2003.

RAVINDRANATH, B. – Some Useful Products from Limonene: A Byproduct of the Citrus Industry. **Journal of Scientific and Industrial Research.** V. 42, p. 82-86, 1983.

REITSEMA, R. H. - **Preparation of *l*-Carvone**. US Patent number US 2802874. Aug. 13 1957.

RODRIGUES, M. I.; IEMMA, A. F. – **Planejamento de Experimentos e Otimização de Processos: uma Estratégia Seqüencial de Planejamentos**. Campinas: Editora Casa do Pão, 2005.

ROHMER, M. – The Discovery of a Mevalonate-Independent Pathway for Isoprenoid Biosynthesis in Bacteria, Algae and Higher Plants. **Natural Product Reports.** V. 16, p. 565-574, 1999.

ROLS, J. L.; CONDORET, J. S.; FONADE, C.; GOMA, G. – Mechanism of Enhanced Oxygen Transfer in Fermentation Using Emulsified Oxygen-Vectors. **Biotechnology and. Bioengineering**. V. 35, n. 4, p. 427–435, 1990.

ROTHENBERGER, O. S.; KRASNOFF, S. B.; ROLLINS, R. B. – Conversion of (+)-Limonene to (–)-Carvone. **Journal of Chemical Education.** V. 57, n.10, p. 741-742, Oct. 1980.

ROYALS, E. E.; HORNE JR., S. E. – Conversion of *d*-Limonene to *l*-Carvone. **Journal of American Chemical Society.** V. 73, p. 5856-5857, Dec. 1951.

ROZENBAUM, H. F.; PATITUCCI, M. L.; ANTUNES, O. A. C.; PEREIRA JR., N. – Production of Aromas and Fragrances Through Microbial Oxidation of Monoterpenes. **Brazilian Journal of Chemical Engineering.** V. 23, n. 3, p. 273-279, 2006.

RUBERTO, G.; BARATTA, M. T. – Antioxidant Activity of Selected Essential Oil Components in Two Lipid Model Systems. **Food Chemistry.** V. 69, p. 167-174, 2000.

SAISUBRAMANIAN, N.; EDWINOLIER, N. G.; NANDAKUMAR, N.; KAMINI, N. R.; PUVANAKRISHNAN, R. – Efficacy of Lipase from *Aspergillus niger* as an Additive in Detergent Formulations: A Statistical Approach. **Journal of Industrial Microbiology and Biotechnology**. V. 33, p. 669-676, 2006.

SAKAMAKI, H.; ITOH, K.; CHAI, W.; HAYASHIDA, Y.; KITANAKA S.; HORIUCHI, C. A. – Biotransformation of (±)-α-Ionone and β-Ionone by Cultured Cells of

Caragana chamlagu. **Journal of Molecular Catalysis B: Enzymatic.** V. 27, n. 4-6, p. 177-181, 2004.

SAKUDA, Y. – The oxidation of Limonene with Selenium Oxide. **Bulletin of Chemical Society of Japan.** v. 42, p. 3348-3349, Nov. 1969.

SANCHEZ-CONTRERAS, A.; JIMENÉZ, M.; SANCHEZ, S. – Bioconversion of Lutein to Products with Aroma. **Applied Microbiology and Biotechnology.** V. 54, n. 4, p. 528-534, 2000.

SANDMANN, G. – Carotenoid Biosynthesis and Biotechnological Application. **Archives of Biochemistry and Biophysics.** V. 385, n. 1, p.4-12, 2001.

SATHE, V. M.; CHAKRAVARTI, K. K.; KADIVAL, M. V.; BHATTACHARYYA, S. C. – Terpenoids. XCIII. Synthesis via Oxidation with Selenium Dioxide. **Indian Journal of Chemistry.** V. 4, n. 9, p. 393-395, 1966.

SAVITHIRY, N.; CHEONG, T. K.; ORIEL, P. – Production of Alpha-Terpienol from *Escherichia coli* Cells Expressing Thermostable Limonene Hydratase. **Applied Biochemistry and Biotechnology.** v. 63, p. 213-220, 1997.

SAVITHIRY, N.; GAGE, D.; FU, W.; ORIEL, P. – Degradation of Pinene by *Bacillus pallidus* BR425. **Biodegradation.** v. 9, n. 5, p. 337-341, Sept. 1998.

SAXENA, R. K.; DAVIDSON, W. S.; SHEORAN, A.; GIRI, B. – Purification and Characterization of an Alkaline Thermostable Lipase from *Aspergillus carneus*. **Process Biochemistry**. V. 39, p. 239-247, 2003.

SCHAEFER, J. P.; HORVATH, B.; KLEIN; H. P. Selenium Dioxide Oxidations. III The oxidation of Olefins. **Journal of Organic Chemistry.** V. 33, n. 7, p. 2647-2655, July 1968.

SCHERER, R.; GODOY, H. T. – Antioxidant Activity Index (AAI) by the 2,2-Diphenyl-1-picrylhydrazyl Method. **Food Chemistry.** V. 112, n. 3, p. 654-658, Feb. 2009.

SCHRADER, J. – Microbial Flavour Production. In: BERGER, R. G. (Ed.) **Flavour and Fragrances: chemistry, bioprocessing and sustainability.** Berlin: Springer-Verlag, 2007, pp. 507-574.

SCHRADER, J.; ETSCHMANN, M. M. W.; SELL, D.; HILMER, J.-M.; RABENHORST, J. – Applied Biocatalysis for the Synthesis of Natural Flavor Compounds – Current Industrial Process and Future Prospects. **Biotechnological Letters.** V. 26, p. 463-472, 2004.

SCHWARTZ, S. H.; QIN, X.; ZEEVAART, J.A.D. Characterization of a Novel Carotenoid Cleavage Dioxygenase from Plants. **The Journal of Biological Chemistry.** V. 276, n. 27, p.25208-25211, 2001.

SCRAGG, A. H. – The Production of Flavours by Plant Cell Cultures. In: BERGER, R. G. (Ed.) **Flavours and Fragrances. Chemistry, Bioprocessing and Sustainability.** 2007, p. 599-614.

SEN, R.; SWAMINATHAN, T. – Response Surface Modeling and Optimization to Elucidate and Analyze the Effects of Inoculum Age and Size on Surfactin Production. **Biochemical Engineering Journal.** V. 21, n. 2, p. 141–148, 2004.

SERRA, S.; FUGANTI, C.; BRENNA, E. – Biocatalytic Preparation of Natural Flavours and Fragrances. **Trends in Biotechnology.** V. 23, n. 4, p. 193-198, 2005.

SEUBERT, W. – Degradation of Isoprenoid Compounds by Microorganisms. I. Isolation and Characterization of an Isoprenoid Degrading Becterium. *Pseudomonas citronellis*, n. sp. **Journal of Bacteriology.** V. 79, p. 426-434, 1960.

SHARMA, R.; CHISTI, Y.; BANERJEE, U. C. – Production, Purification, Characterization, and Applications of Lipases. **Biotechnology Advances**. V. 19, p. 627-662, 2001.

SHARMA, R.; SONI, S. K.; VOHRA, R. M.; GUPTA, L. K.; GUPTA, J. K. – Purification and Characterization of a Thermostable Alkaline Lipase From a New Thermophilic *Bacillus* sp. RSJ-1. **Process Biochemistry**. V. 37, p. 1075-1084, 2002.

SHAW, P. E. – Review of Quantitative Analysis of Citrus Essential Oils. **Journal of Agricultural and Food Chemistry**. V. 27, p. 246-257, 1979.

SHEPPARD, J. D.; MULLIGAN, C. N. – The Production of Surfactin by *Bacillus subtilis* Grown on Peat Hydrolyzate. **Applied Microbiology and Biotechnology**. V. 27, n. 2, p. 110-116, 1987.

SHIMADA, T.; SHINDO, M.; MIYAZAWA, M. – Species Differences in the Metabolism of (+) and (–)-Limonene and Their Metabolites, Carveols and Carvones, by Cytochrome P450 Enzymes in Liver Microsomes of Mice, Rats, Guinea Pigs, Rabbits, Dogs, Monkeys and Humans. **Drug Metabolism and Pharmacokinetics.** V. 17, n. 6, p. 507-515, 2002.

SHUKLA, O. P.; BHATTACHARYYA, P. K. – Microbiologica Transformation of Terpenes. XI. Pathways of Degradation of α- and β-pinenes in a soil *Pseudomonad* (PL-strain). **Indian Journal of Biochemistry.** V. 5, p. 92-101, 1968.

SHUKLA, O. P.; MOHOLAY, M. N.; BHATTACHARYYA, P. K. – Microbiologica Transformation of Terpenes. X. Fermentation of α- and β-Pinenes. **Indian Journal of Biochemistry.** V. 5, p. 79-91, 1968.

SINGH, K. L.; TANDON, S.; KAHOL, A. P.; AGGARWAL, K. K.; KUMAR, S. – Terpineol: An Aroma Chemical of Value in the Food, Flavour and Fragrance Industry. **Journal of Medicinal and Aromatic Plant Science**. V. 20, p. 779-786, 1998.

SKEHAN, P. R.; SCUDIERO, D. – New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening . **Journal of the National Cancer Institute.** V. 82, n. 13, p. 1107-1118, 1990.

SKROBOT, F. C.; VALENTE; A. A.; NEVES, G.; ROSA, I.; ROCHA, J.; CAVALEIRO, J. A. S. – Monoterpenes Oxidation in the Presence of Y Zeolite-Entrapped Manganese(III) Tetra(4-*N*-Benzylpyridyl)Porphyrin. **Journal of Molecular Catalysis A: Chemical.** V. 201; n. 1–2; p. 211-222; July 2003.

SOWDEN, R. J.; YASMIN, S.; REES, N. H.; BELL, S. G.; WONG, L.-L. – Biotransformation of the Sesquiterpene (+)-Valencene by Cytochrome $P450_{cam}$ and $P450_{BM-3}$. **Organic and Biomolecular Chemistry.** V. 3, p. 57-64, 2005.

SPEELMANS, G.; BIJLSMA, A.; EGGINK, G. – Limonene Bioconversion to High Concentrations of a Single and Stable Product, Perillic Acid, by a Solvent-resistant *Pseudomonas putida* Strain. **Applied Microbiology and Biotechnology.** v. 50, n. 5, p. 538-544, Nov. 1998.

STALLCUP, W. D.; HAWKINS, J. E. – Reactions of α-Pinene. I. With Selenium Dioxide in Various Solvents. **Journal of the American Chemical Society.** V. 63, p. 3339-3341, 1941.

STALLCUP, W. D.; HAWKINS, J. E. – Reactions of α-Pinene. II. With Selenium Dioxide in Acetic Acid. **Journal of the American Chemical Society.** V. 64, p. 1807-1809, 1942.

STEFANOV, S.; YANKOV, D.; BESCHKOV, V. – Biotransformation of Phytosterols to Androstenedione in Two Phase Water-Oil Systems. **Chemical and Biochemical Engineering Quarterly**. V. 20, n. 4, p. 421-427, 2006.

SUGA, T.; HIRATA, T. – Biotransformation of Exogenous Substrates by Plant Cells. **Phytochemistry.** V. 29, n. 8, p. 2393-2406, 1990.

SUMIMOTO, M.; SUZUKI, T.; KONDO, T. – Oxidation of D-limonene with Selenium Dioxide-Hydrogen Peroxide. **Agricultural and Biological Chemistry.** V. 38, n. 5, p. 1061-1065, 1974.

SWIFT, K. A. D. – Catalytic Transformations of the Major Terpene Feedstocks. **Topics in Catalysis.** V. 27, n. 1-4, p. 143-155, 2004.

TAKECHI, H.; MIYAZAWA, M. – Biotransformation of Geraniol by the Larvae of Common Cutworm (*Spodoptera litura*). **Journal of Oleo Science.** V. 55, n. 3, p. 143-149, 2006.

TAN, Q.; DAY, D. F. – Bioconversion of Limonene to α-Terpineol by Immobilized *Penicillium digitatum*. **Applied Microbiology and Biotechnology.** v. 49, n. 1, p. 96-101, 1998a.

TAN, Q.; DAY, D. F. – Organic Co-solvent Effects on the Bioconversion of (R)-(+)-Limonene to (R)-(+)-α-Terpineol. **Process Biochemistry.** v. 33, n. 7, p. 755-761, 1998b.

TAN, Q.; DAY, D. F.; CADWALLADER, K. R. – Bioconversion of (R)-(+)-Limonene by *P. digitatum* (NRRL 1202). **Process Biochemistry.** v. 33, n. 1, p. 29-37, 1998.

TECELÃO, C. S. R.; VAN KEULEN, F.; DA FONSECA, M. M. R. – Development of a Reaction System for the Selective Conversion of (−)-*trans*-Carveol to (−)-Carvone with Whole Cells of *Rhodococcus erythropolis* DCL14. **Journal of Molecular Catalysis B: Enzymatic.** V. 11, n. 4-6, p. 719-724, Jan. 2001.

TEISSEIRE, P. J. – **Chemistry of Fragrant Substances.** Translated by Peter A. Cadby. New York: VCH Publishers, 1994. 458 p.

THOMAS, A. F.; BUCHER, W. – Menthatrienes and the oxidation of limonene. **Helvetica Chimica Acta.** V. 53, n. 4, p. 770-775, 1970.

TILDEN, W. A.; LOND, F. C. S. – On the Action of Nitrosyl Chloride on Organic Bodies. Part II. On Turpentine Oil. **Journal of the Chemical Society.** V. 28, p.514-519, 1875.

TILDEN, W. A.; SHENSTONE, W. A. – Isomeric Nitroso-Terpenes. **Journal of the Chemical Society.** V. 31, p.554-561, 1877.

TONIAZZO, G.; DE OLIVEIRA, D.; DARIVA, C.; OESTREICHER, E. G.; ANTUNES, O. A. C. – Biotransformation of (–)β-pinene by *Aspergillus niger* ATCC 9642. **Applied Biochemistry and Biotechnology.** V. 121-124, p. 837-844, 2005.

TRACHTENBERG, E. N.; CARVER, J. R. – Stereochemistry of Selenium Dioxide Oxidation of Cyclohexenyl Systems. **Journal of Organic Chemistry.** v. 35, n. 5, 1646-1653, May 1970.

TRACHTENBERG, E. N.; NELSON, C. H.; CARVER, J. R. – Mechanism of Selenium Dioxide Oxidation of Olefins. **Journal of Organic Chemistry.** v. 35, n. 5, 1653-1658, May 1970.

TRUDGILL, P. W. – Microbial Metabolism of Monoterpenes – Recent Developments. **Biodegradation.** V. 1, n. 2-3, p. 93-105, June 1990.

TRUDGILL, P. W. – Terpenoid Metabolism by *Pseudomonas*. In: The Bacteria. A Treatise on Structure and Function. Vol. X. San Diego: Academic Press, Inc. GUNSALUS, I. C. (Cons. Ed.); SOKATCH, J. R.; ORNSTON, L. N. (Ed.-in-Chief). p. 483-525, 1986.

TRYTEK, M.; FIEDUREK, J. – A Novel Psychrotrophic Fungus, *Mortierella minutissima*, for D-limonene Biotransformation. **Biotechnology Letters.** V. 27, n. 3, p. 149-153, Feb. 2005.

TRYTEK, M.; FIEDUREK, J. – Biotransformation of D-limonene to Carvone by Means of Glucose Oxidase and Peroxidase. **Acta Microbiologica Polonica.** V. 51; n. 1; p. 57–62; 2002.

TRYTEK, M.; FIEDUREK, J.; POLSKA, K.; RADZKI, S. – A Photoexcited Porphyrin System as a Biomimetic Catalyst for D-Limonene Biotransformation. **Catalysis Letters.** V. 105; n. 1–2; p. 119–126; Nov. 2005.

TUDROSZEN, N. J.; KELLY, D. P.; MILLIS, N. F. – α-Pinene Metabolism by *Pseudomonas putida*. **The Biochemical Journal.** V. 168, n. 2, p. 315-318, Nov. 1977.

UNGER, B. P.; SLIGAR, S. G; GUNSALUS, I. C. – *Pseudomonas* Cytochromes *P*-450. In: GUNSALUS, I. C. (Cons. Ed.); SOKATCH, J. R.; ORNSTON, L. N. (Ed.-in-Chief) **The Bacteria. A Treatise on Structure and Function.** San Diego: Academic Press, Inc, Vol. X, 1986, p. 557-589.

URIBE, S.; PEÑA, A. – Toxicity of Allelopathic Monoterpene Suspensions on Yeasts. Dependence on the Droplet Size. **Journal of Chemical Ecology**. V. 16, n. 4, p. 1399-1408, 1990.

VALKO, M.; IZAKOVIC, M.; MAZUR, M.; RHODES, C. J.; TELSER, J. – Role of Oxygen Radicals in DNA Damage and Cancer Incidence. **Molecular and Cellular Biochemistry.** V. 266, p. 37-56, 2004.

VAN BEILEN, J. B.; HOLTACKERS, R.; LÜSCHER, D.; BAUER, U.; WITHOLT, B.; DUETZ, W. A.– Biocatalytic Production of Perillyl Alcohol from Limonene by Using a Novel *Mycobacterium* sp. Cytochrome P450 Alkane Hydroxylase Expressed in *Pseudomonas putida*. **Applied and Environmental Microbiology.** V. 71, n. 4, p. 1737-1744, Apr. 2005.

VAN DER WERF, M. J.; DE BONT J., A. M.; LEAK, D. J. Opportunities in Microbial Biotransformation of Monoterpenes. **Advances in Biochemical Engineering, Biotechnology.** V. 55, p. 147-177, 1997.

VAN DER WERF, M. J.; KEIJZER, P. M.; VAN DER SCHAFT, P. H. –*Xanthobacter* sp. C20 Contains a Novel Bioconversion Pathway for Limonene. **Journal of Biotechnology.** V. 84, n. 2, p. 133-143, Nov 2000.

VAN DER WERF, M. J.; SWARTS, H. J.; DE BONT, J. A. M. – *Rhodococcus erythropolis* DCL14 Contains a Novel Degradation Pathway for Limonene. **Applied and Environmental Microbiology.** V. 65, n. 5, p. 2092-2102, May 1999.

VAN DYK, M. S.; VAN RENSBURG, E.; MOLELEKI, N. – Hydroxylation of (+)-Limonene, (–)α-Pinene and (–)β-Pinene by a *Hormonema* sp. **Biotechnology Letters.** V. 20, n. 4, p.431-436, 1998.

VAN DYK, M. S.; VAN RENSBURG, E.; RENSBURG, I. P. B.; MOLELEKI, N. – Biotransformation of Monoterpenoid Ketones by Yeasts and Yeast-like Fungi. **Journal of Molecular Catalysis B: Enzymatic.** V. 5, p.149-154, 1998.

VAN RENSBURG, E.; MOLELEKI, N.; VAN DER WALT, J. P.; BOTES, P. J.; VAN DYK, M. S. – Biotransformation of (+)-Limonene and (–)-Piperitone by Yeasts and Yeast-like Fungi. **Biotechnology Letters.** V. 19, n. 8, p.779-782, 1997.

VANĚK, T.; HALÍK, J.; VAŇKOVÁ, R.; VALTEROVÁ, I. – Formation of *trans*-Verbenol and Verbenone from α-Pinene Catalysed by Immobilised *Picea abies* Cells. **Bioscience, Biotechnology and Biochemistry.** V. 69, n. 2, p. 321-325, 2005.

VANĚK, T.; VALTEROVÁ, I.; VAŇKOVÁ, R.; VAISAR, T. – Biotransformation of (–)-Limonene Using *Solanum aviculare* and *Dioscorea deltoidea* Immobilized Plant Cells. **Biotechnology Letters.** v. 21, p. 625-628, 1999.

VOIGT, C. A.; SCHAEFER, W.; SALOMON, S. A Secreted Lipase of *Fusarium graminearum* is a Virulence Factor Required for Infection of Cereals. **Plant Journal.** V. 42, n. 3, p. 364-375, 2005.

WACHÉ, Y.; RATULD, A.B.; BELIN, J.M. Dispersion of β-Carotene in Processes of Production of β-ionone by Cooxidation using Enzyme-Generated Reactive Oxygen Species. **Process Biochemistry.** V. 41, n. 11, p. 2337-2341, 2006.

WAGNER, K.-H.; ELMADFA, I. – Biological Relevance of Terpenoids. **Annals of Nutrition and Metabolism.** V. 47, p. 95-106, 2003.

WAGNER, R. – **Composição de Voláteis e Aroma de Salames Nacionais Tipos Italiano e Milano.** Campinas: Unicamp, 2008 (Tese – Doutorado em Ciência de Alimentos).

WAGNER, R.; BARROS, F.F.C.; BICAS, J.L.; GODOY, H.T.; PASTORE, G.M. **Método para análise da biotransformação de limoneno em α-terpineol por microextração em fase sólida.** Trabalho apresentado ao 7° Simpósio Latinoamericano de Ciência e Tecnologia de Alimentos (SLACA), Campinas, 2007. Publicado em anais em formato eletrônico.

WANG, T. – Soybean Oil. In: GUNSTONE, F. D. (Ed.) **Vegetable Oils in Food Technology. Composition, Properties and Uses**. Oxford: Blackwell Publishing Ltd, ch. 2, 2002, pp. 18-58.

WELSH, F. W.; MURRAY, W. D.; WILLIAMS, R. E. – Microbiological and Enzymatic Production of Flavor and Fragrance Chemicals. **Critical Reviews in Biotechnology.** V. 9, n. 2, p. 105-169, 1989.

WIBERG, K. B.; NIELSEN, S. D. – Some Observations on Allylic Oxidation. **Journal of Organic Chemistry.** V. 29, n. 11, p. 3353-3361, 1964.

WILSON, C. W.; SHAW, P. E. – (+) Limonene Oxidation with Selenium Dioxide-Hydrogen Peroxide. **Journal of Organic Chemistry.** v. 38, n. 9, 1664-1687, Apr. 1973.

WINTERHALTER, P.; ROUSEFF, R. Carotenoid-Derived Aroma compounds: An Introduction. In: WINTERHALTER, P.; ROUSEFF, R. (Eds.) Carotenoid-derived aroma compounds. Washington, DC: American Chemical Society. ACS Symposium Series 802, Vol. 219. 2001, pp. 1-17.

WOLKEN, W. A. M.; VAN DER WERF, M. J. – Geraniol Biotransformation-Pathway in Spores of *Penicillium digitatum*. **Applied Microbiology and Biotechnology.** V. 57, n. 5-6, p. 731-737, Dec. 2001.

WOOTTON, R. C. R.; FORTT, R.; DE MELLO, A. J. – A Microfabricated Nanoreactor for Safe, Continuous Generation and Use of Singlet Oxygen. **Organic Process Research and Development.** V. 6, n. 2, p. 187-189, 2002.

WRIGHT, S. J.; CAUNT, P.; CARTER, D.; BAKER, P. B. – Microbial Oxidation of Alpha-Pinene by *Serratia marcescens*. **Applied Microbiology and Biotechnology.** V. 23, n. 3-4, p.224-227, 1986.

WU, Z.; ROBINSON, D. S.; HUGHES, R. K.; CASEY, R.; HARDY, D.; WEST, S. I. – Co-oxidation of β-Carotene Catalyzed by Soybean and Recombinant Pea Lipoxygenases. **Journal of Agricultural and Food Chemistry.** V. 47, n. 12, p. 4899-4906, Dec. 1999.

WÜST, M.; LITTLE, D. B.; SCHALK, M.; CROTEAU, R. – Hydroxylation of Limonene Enantiomers and Analogs by Recombinant (–)-Limonene 3- and 6-Hydroxylases from Mint (*Mentha*) Species: Evidence for Catalysis within Sterically Constrained Active Sites. **Archives of Biochemistry and Biophysics.** V. 387, p. 125-136, 2001.

XU, M.; FLOYD, H.S.; GRETH, S.M.; CHANG, W.-C.L.; K. LOHMAN, STOYANOVA, R.; KUCERA, G.L.; KUTE, T.E.; WILLINGHAM, M.C.; M.S. MILLER – Perillyl Alcohol-Mediated Inhibition of Lung Cancer Cell Line Proliferation: Potential Mechanisms for its Chemotherapeutic Effects. **Toxicology and Applied Pharmacology.** V. 195, n. 2, p. 232-246, 2004.

YASSIN, A. A.; MOHAMED, I. O.; IBRAHIM, M. N.; YUSOFF, M. S. – Effects of Enzymatic Interesterification on Melting Point of Palm Olein. **Applied Biochemistry and Biotechnology**. V. 110, p. 45-52, 2003.

YERUVA, L.; PIERRE, K.J.; ELEGBEDE, A.; WANG, R.C.; CARPER S.W. – Perillyl Alcohol and Perillic Acid Induced Cell Cycle Arrest and Apoptosis in Non Small Cell Lung Cancer Cells. **Cancer Letters**. V. 257, p. 216–226, 2007.

YOO, S. K.; DAY, D. F. – Bcaterial Metabolism of α- and β-Pinene and Related Monoterpenes by *Pseudomonas* sp. Strain PIN. **Process Biochemistry.** V. 37, p. 739-745, 2002.

YOO, S. K.; DAY, D. F.; CADWALLADER, K. R. – Bioconversion of α- and β-Pinene by *Pseudomonas* sp. Strain PIN. **Process Biochemistry.** V. 36, p. 925-932, 2001.

YURI, T.; DANBARA, N.; TSUJITA-KYUTOKU, M.; KIYOZUKA, Y.; SENZAKI, H.; SHIKATA, N.; KANZAKI, H.; TSUBURA, A. – Perillyl Alcohol Inhibits Human Breast Cancer Cell Growth *in vitro* and *in vivo*. **Breast Cancer Research and Treatment**. V. 84, p. 251–260, 2004.

YUSTE, L.; CANOSA, I.; ROJO, F. – Carbon-Source-Dependent Expression of the *PalkB* Promoter from the *Pseudomonas oleovorans* Alkane Degradation Pathway. **Journal of Bacteriology.** V. 180, p. 5218-5226, 1998.

ZHENG, Y.; LU, L. – Synthesis of Perillaldehyde Oxime from α-Pinene. **Xiangtan Daxue Ziran Kexue Xuebao.** V. 17, n. 1, p. 58-61, 1995.

ZHU, W.; ASGHARI, G.; LOCKWOOD, G. B. – Factors Affecting Volatile Terpene and Non-Terpene Biotransformation Products in Plant Cell Cultures. **Fitoterapia.** V. 71, p. 501-506, 2000.

ZORN, H.; LANGHOFF, S.; SCHEIBNER M.; BERGER, R. G. – Cleavage of β,β-Carotene to Flavor Compounds by Fungi. **Applied Microbiology and Biotechnology.** V. 62, p. 331-336, 2003a.

ZORN, H.; LANGHOFF, S.; SCHEIBNER M., NIMTZ, M.; BERGER, R.G. – A Peroxidase from *Lepista irina* Cleaves β,β-Carotene to Flavor Compounds. **Biological Chemistry.** V. 384, p. 1049-1056. 2003b.

ZORN, H.; NEUSER, F.; BERGER, R. G. – Degradation of α-Pinene Oxide and [$^2H_7$]-2,5,6-Trimethyl-hept-(2*E*)-enoic Acid by *Pseudomonas fluorescens* NCIMB 11761. **Journal of Biotechnology.** V. 107, p. 255-263, 2004.